UNIVERSIDAD AUSTRAL DE CHILE
ESCUELA DE GRADUADOS/SEDE PUERTO MONTT

STUDY OF THE TYPE 4B PROTEIN SECRETION SYSTEM (Dot/Icm) AND THE EFFECTOR PROTEIN SdhA IN *Piscirickettsia salmonis.*

DOCTORAL THESIS

MARCOS ANDRÉS CORTÉS SOBARZO
PUERTO MONTT – CHILE

2015

STUDY OF THE TYPE 4B PROTEIN SECRETION SYSTEM (Dot/Icm) AND THE EFFECTOR PROTEIN SdhA IN *Piscirickettsia salmonis.*

Thesis work presented to Escuela de Graduados - Sede Puerto Montt Universidad Austral de Chile, in order to complete all the requirements to get the PhD degree in Aquaculture Sciences

By

MARCOS ANDRÉS CORTÉS SOBARZO

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INFORME DE APROBACIÓN DE TESIS DE DOCTORADO

The Thesis Evaluation Committee notifies to Dr. José Luis Iriarte, Head Department of Escuela de Graduados Sede Puerto Montt, that the Doctoral Thesis Work presented by the applicant

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each lane was 80 μg. Silver staining of the same samples was used as a loading control (data not shown).

FIGURE 20 Schematic model of the distribution of GSP and T4BSS proteins in *P. salmonis* with the possible substrate using proposed models by: Isberg, *et al.*, 2009; Schulze, *et al.*, 2013; van Schaik, *et al.*, 2013. 65

ABREVIATIONS INDEX

ASO	Antisense Oligonucleotide
ATP	Adenosin Tri Phosphate
BCA	Bicinchoninic Acid
BKD	Bacterial Kidney Disease
BSA	Bovine Serum Albumin
cDNA	complementary Deoxynucleic Acid
°C	Celsius degree
d	days
DAPI	4',6- diamidino-2-phenylindole
Dot/Icm	Defect Organelle Trafficking/ Intracellular Multiplication
EEA-1	Early Endosome Antigen 1
EPEC	Enteropathogenic *E. coli*
FBS	Fetal Bovine Serum
GSP	General Secretion Pathway
h	hour
HRP	Horse Radish Peroxidase
IFAT	Immunofluorescent Antibody
IgY	Immunoglobulin Y
IFOP	Fisheries Development Institute
IM	Intramuscular
i.p	Intra peritoneal
IPNV	Infectious Pancreatic Necrosis Virus
ISA	Infectious Salmon Anemia
kDa	KiloDalton
L	liter
L-15	Leibovitz's Medium L-15
LAMP	Lysosome Associated Membrane Protein

LCV	*Legionella* Containing Vacuole
M	Molar
mA	MiliAmpers
mg	milligrams
mL	milliliter
MOI	Multiplicity of Infection
mRNA	messenger RiboNucleic Acid
nm	nanometer
N	Normal
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PMSF	Phenyl Methyl Sulphonyl Fluoride
P. salmonis	*Piscirickettsia salmonis*
Rab	Ras-related in brain GTPases
RILP	Rab Interacting Lysosomal Protein
RIPA	Radio Immunoprecipitation Assay buffer
rRNA	ribosomal RiboNucleic Acid
SDS	Sodium Dodecyl Sulfate
SHK-1	Salmon Head Kidney
SRS	Salmon Rickettsial Syndrome
T1SS	Type 1 Secretion System
T2SS	Type 2 Secretion System
T3SS	Type 3 Secretion System
T4SS	Type 4 Secretion System
T4BSS	Type 4B Secretion System
T5SS	Type 5 Secretion System
T6SS	Type 6 Secretion System
T7SS	Type 7 Secretion System
T9SS	Type 9 Secretion System

TBS	Tris Buffered Saline
µg	microgram
µL	microliter
µm	micrometer
USD$	United States Dollars
v/v	volume/volume
w/v	weight/volume

SUMMARY

Chilean Salmon Farming is an important industry nowadays in Chile with significant economics entry to the country. The troubles with the handling and maintenance of this industry to avoid product losses is a key factor of concern right now. Piscirickettsiosis disease has been a problem that persist over the time in this industry and several attempts had been made to try to solve the mortalities induced by this fish pathogen. According to Aquaculture and Fishery report from Sernapesca, the amount of harvest Chilean Atlantic salmon was around 152.806 tons in 2014 and represent around USD$ 1,292 million. During the year 2014, the main cause of mortalities is Piscirickettsiosis, affecting all three salmonid species farmed in Chile, reaching 74% in Atlantic salmon, 73% in rainbow trout and 36% in coho salmon. The lack of effective treatments to control Piscirickettsiosis has emphasized the need to develop techniques for diseases prevention. Management of the disease is based on several husbandry practices including the application of immunostimulants of unproven efficacy and the control of vertical transmission by an expensive selection procedure during reproduction. *Piscirickettsia salmonis* is the causative agent for Piscirickettsiosis diseases that affects the three salmonid species farming in Chile. Currently, our knowledge about this pathogen is limited to a few articles that described the induction of apoptosis in phagocytic host cells, the presence of the type 4B secretion system at transcript level, the possible evasion of the phagocytic pathway after internalization in the host cell, the increased gene expression levels of two virulence factors with a key role in the intracellular survival (ClpB and BipA), and recently the enclosed genome for the LF-89 strain.

The results found in this work, could provide a real evidence how the bacteria can establish the infection inside the host cell, using the Type 4B Secretion System to translocate effector proteins that subvert the vesicle trafficking to its own benefits. Showing the presence at protein level of this secretion system, could generate the possibility to use new antigens to develop new vaccines against this pathogen to control the disease and lower the mortality induced by *P. salmonis*.

RESUMEN

La Salmonicultura chilena es una industria importante hoy en día en el país con un ingreso económico significativo. Los problemas con el manejo y mantenimiento de este sector para evitar pérdidas de producto por enfermedades es un factor clave de preocupación en este momento. La enfermedad Piscirickettsiosis ha sido un problema que persiste en el tiempo en esta industria y se han realizado varios intentos para tratar de resolver las mortalidades inducidas por este agente patógeno de peces. Según informe de Sernapesca, la cantidad de cosecha de salmón Atlántico chileno fue de alrededor de 152.806 toneladas en 2014 y representan alrededor de USD $ 1.292 millones. Durante el año 2014, la principal causa de mortalidad es Piscirickettsiosis, que afectó a las tres especies de salmónidos cultivados en Chile, alcanzando el 74% en el salmón del Atlántico, el 73% en la trucha arco iris y el 36% en el salmón Coho. La falta de tratamientos efectivos para controlar la Piscirickettsiosis ha hecho hincapié en la necesidad de desarrollar técnicas para la prevención de enfermedades. El manejo de la enfermedad se basa en varias prácticas durante el cultivo, incluyendo la aplicación de inmunoestimulantes de eficacia no comprobada y el control de la transmisión vertical por un procedimiento de selección caro durante la reproducción. *Piscirickettsia salmonis* es el agente causante de la enfermedad Piscirickettsiosis que afecta a las tres especies de salmónidos cultivados en Chile. Actualmente, nuestro conocimiento acerca de este patógeno se limita a unos pocos artículos que describen la inducción de apoptosis en células huésped fagocítica, la presencia del sistema de secreción de tipo 4B a nivel génico, la posible evasión de la vía fagocítica después de la internalización en la célula huésped, altos niveles de expresión de genes de dos factores de virulencia con un papel clave en la supervivencia intracelular (ClpB y BIPA), y recientemente el genoma cerrado para la cepa tipo LF-89. Los resultados encontrados en este trabajo, proporcionan evidencia real de como este patógeno puede establecer la infección dentro de la célula huésped, mediante el Sistema de Secreción Tipo 4B para traslocar proteínas efectoras (SdhA) que subvierten el tráfico vesicular para su propio beneficio. Demostrando la presencia a nivel proteico de este sistema de secreción se podrían generar vacunas contra este patógeno, utilizando estos nuevos antígenos para controlar la enfermedad y reducir la mortalidad generada por *P. salmonis*.

1. INTRODUCTION.

1.1 CHILEAN SALMON FARMING

The beginnings of salmon farming in Chile are dated on the year 1850 when foreign investors and the Fisheries Development Institute (IFOP) introduced exotic aquatic species; the first Coho salmon was imported to Chile in 1921. In 1974 began the commercial Rainbow trout farming and also the exportation, which triggered a radical shift to salmon farming in Chile. In 1978 the Chilean government contribution was the establishment of the Fisheries Department and the National Fisheries Service (*Servicio Nacional de Pesca y Acuicultura, SERNAPESCA*), and between 1978 and 1980 several private initiatives lead to the foundation of exclusively salmon farming companies. In 1990 the first Coho salmon was cultivated. This step represents the first scientific advancement regarding fish industries in Chile and triggered the real takeoff for salmon farming. Together with the improvement of feeding techniques, the industry began to use better farming processes to improve the rearing of salmon. In 2003 the industry developed a Code of Good Practice but in 2007 the first case of Infectious Salmon Anemia (ISA) was reported. This disease caused important economics losses to the industry and a rapid setting up of a public-private partnership was required to solve this problem. Currently, the three salmonid species cultures in Chile are Atlantic salmon, the most commercial species, reaching a weight to 4,5- 5 kgs when harvested; Pacific salmon which takes 10 to 12 months to harvest with almost 3 kgs of weight; and Rainbow trout which also is harvested around 3 kgs taking 10 to 12 months to reach this weight (salmonchile.cl). According to Aquaculture and Fishery report from Sernapesca (2014), the amount of harvested Chilean Atlantic salmon was around 152.806 tons in 2014 and represent around USD$ 1,292 million (fig. 1). During the year 2014, the main cause of mortalities is Piscirickettsiosis, affecting all three salmonid species farmed in Chile, reaching 74% in Atlantic salmon, 73% in Rainbow trout and 36% in Coho salmon (fig. 2) (Sanitary report from Sernapesca 2014/February 2015).

Total export in aquaculture sector until May from 2013 to 2014				
	Export Value (mUSD$)		Quantity (tons)	
Resource	2013	2014	2013	2014
Atlantic salmon	826.676	1.292.661	127.524	152.806
Coho salmon	253.983	378.435	76.287	62.318
Rainbow trout	334.587	322.496	58.290	32.567

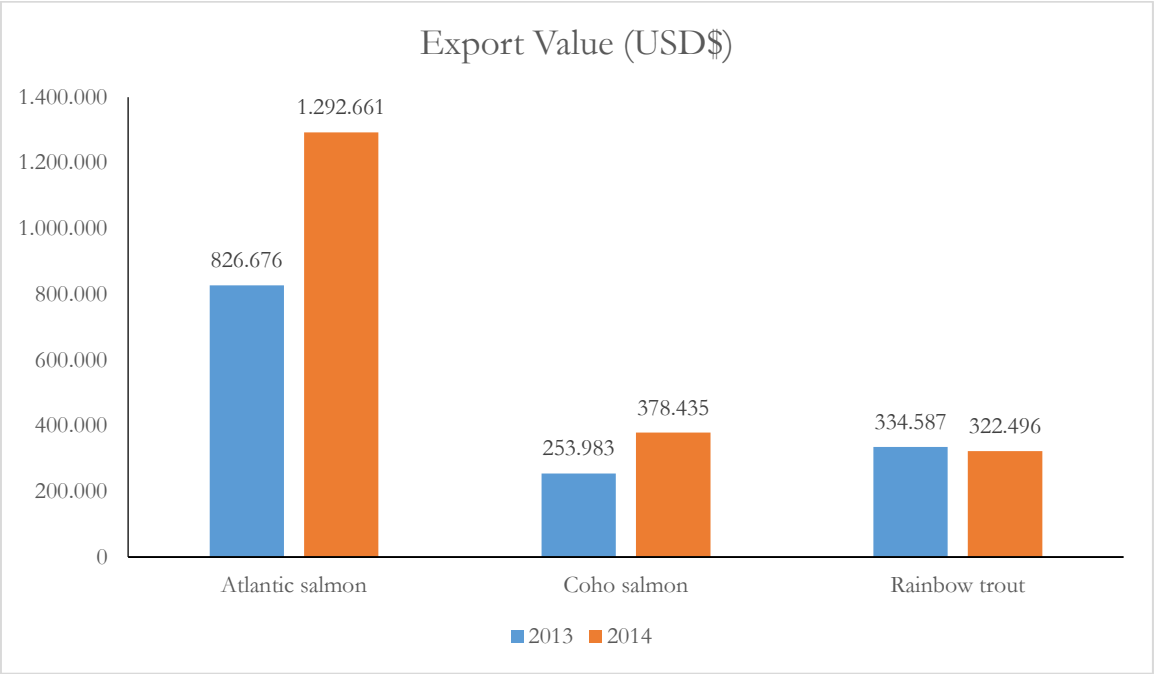

Figure 1: Harvest amount (tons) and economics export (USD$) of three salmonid species cultured in Chile during 2013- 2014 (Sernapesca 2014).

1.2 PATHOGENS AND DISEASE IN CHILEAN SALMON FARMING

Aquaculture is nowadays an emerging and very productive industry in Chile, specifically, salmon farming. With the increased development of the industry, the outcome of several diseases is inevitable. Handling and good practice are the key factors to avoid the beginning and dissemination of the diseases.

There are many microorganisms that induce several diseases in salmonid species. For viral infection there are two main viruses inducing diseases in salmonid fishes: the Infectious Pancreatic Necrosis Virus (IPNV) and Infectious Salmon Anemia Virus (ISAV). The ISAV induced a significant economic loses in 2007 spreading among several companies of salmon farming in Chile. Currently there are other kinds of viral infection in Chilean salmon farming but not with the severity of ISAV but still its needed to be considered (Crane and Hyatt, 2011). For Gram positive bacterial infection, among others, there are Bacterial Kidney Disease (BKD) induced by *Renibacterium salmoninarum*, Furunculosis induced mainly by *Flavobacterium salmoninarum*, Streptococcosis induced by *Streptococcus phocae* and Mycobacteriosis induced by *Mycobacterium marinum*. For Gram negative bacterial infection there are Vibriosis induced mainly by *Vibrio anguillarum*, winter ulcer induced by *Moritella viscosa*, Photobacteriosis (pasteurellosis) induced by *Photobacterium damselae subsp. piscicida*, Furunculosis induced by *Aeromonas salmonicida*, Edwardsiellosis induced by *Edwardsiella tarda*, enteric redmouth induced by *Yersinia ruckeri* and Piscirickettsiosis induced by the intracellular pathogen *Piscirickettsia salmonis* (Toranzo *et al.*, 2005; Sudheesh *et al.,* 2012). *Piscirickettsia salmonis* is the intracellular bacterium that causes Salmonid Rickettsial Syndrome (SRS) or Piscirickettsiosis, an infectious disease that kills millions of farmed fish each year. Piscirickettsiosis is a serious threat to the global salmon aquaculture industry (Rise, *et al.*, 2004).

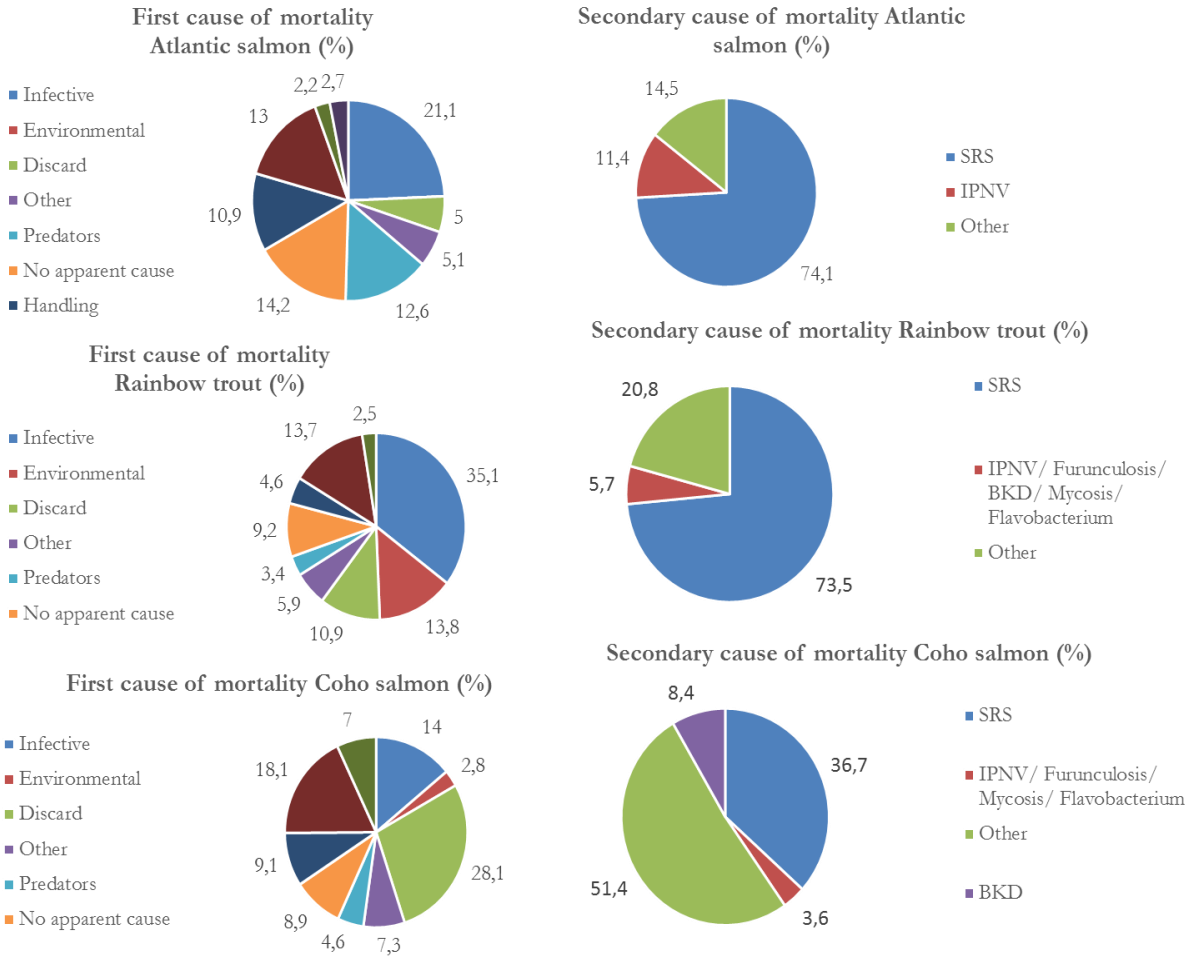

Figure 2: accumulative mortalities induced by infectious diseases affecting the three salmonid species farmed in Chile, according to sanitary report from Sernapesca 2014. Piscirickettsiosis is the main cause of mortalities in Atlantic salmon and Rainbow trout.

1.2.1 PISCIRICKETTSIOSIS AND *Piscirickettsia salmonis*

Piscirickettsiosis (fig. 3) is a systemic disease that shows external clinical signs as pale gills from significant anemia, fin ulcerations, abdominal swelling, petechial and ecchymotic hemorrhages on the base of fins and in the perianal zones. Also, the fish behavior change when they are severely affected with this disease, showing dark skin color, inappetence, lethargy and swim near to the surface or edges of cages. Internal clinical signs typical are swollen kidney, liver and spleen due to the systemically spread of the bacterium. Also it is observed petechial hemorrhages in visceral organs, swim bladder wall and skeletal muscle, and also the presence of caverns inside the muscle as a clear area or with exudates. (Rozas and Enriquez, 2014). Piscirickettsiosis occurs during saltwater stage of the salmonid life cycle, and is horizontally transmitted (Gaggero *et al.*, 1995). The etiological agent that causes this disease is *Piscirickettsia salmonis* characterized as a Gram-negative, intracellular facultative bacterium. It is described as pleomorphic, predominantly coccoid in shape, pairs grouped, ranging in diameter between 0.5 to 1.5 μm (fig. 4), and it has been present for a long time and apparently since the Salmon farming industry begun in Chile. It was first described in the year 1989 when the bacterium was considered to be a *Rickettsia* species and classified as an obligate intracellular microorganism. Currently we know that this bacterium can grow in absence of cells in specialized medium. Indeed, Yáñez *et al.* (2012) described a specific medium in which the bacteria can grow perfectly in a range between 15 to 18 °C. This bacterium, isolated in 1989 from moribund Coho salmon from saltwater net pen site in the south of Chile, was the first Rickettsia-like organism (RLO) recognized as a fish pathogen (Fryer *et al.*, 1990). The *Piscirickettsiaceae* family currently contains five genera, *Piscirickettsia, Cycloclasticus, Hydrogenovibrio, Methylophaga* and *Thiomicrospira*, that are phylogenetically related but they have a few phenotypic characteristics in common and the only member of the *Piscirickettsia* genus is *Piscirickettsia salmonis* (Fryer and Hedrick, 2003). Molecular phylogenetic analysis based on sequencing the 16S rRNA gene, placed *Piscirickettsia salmonis* in the new family of *Piscirickettsiaceae* within the class of γ- proteobacteria, most closely related to *Coxiella, Francisella* and *Legionella* (Fryer *et al.*, 1992; Rozas and Enriquez, 2014).

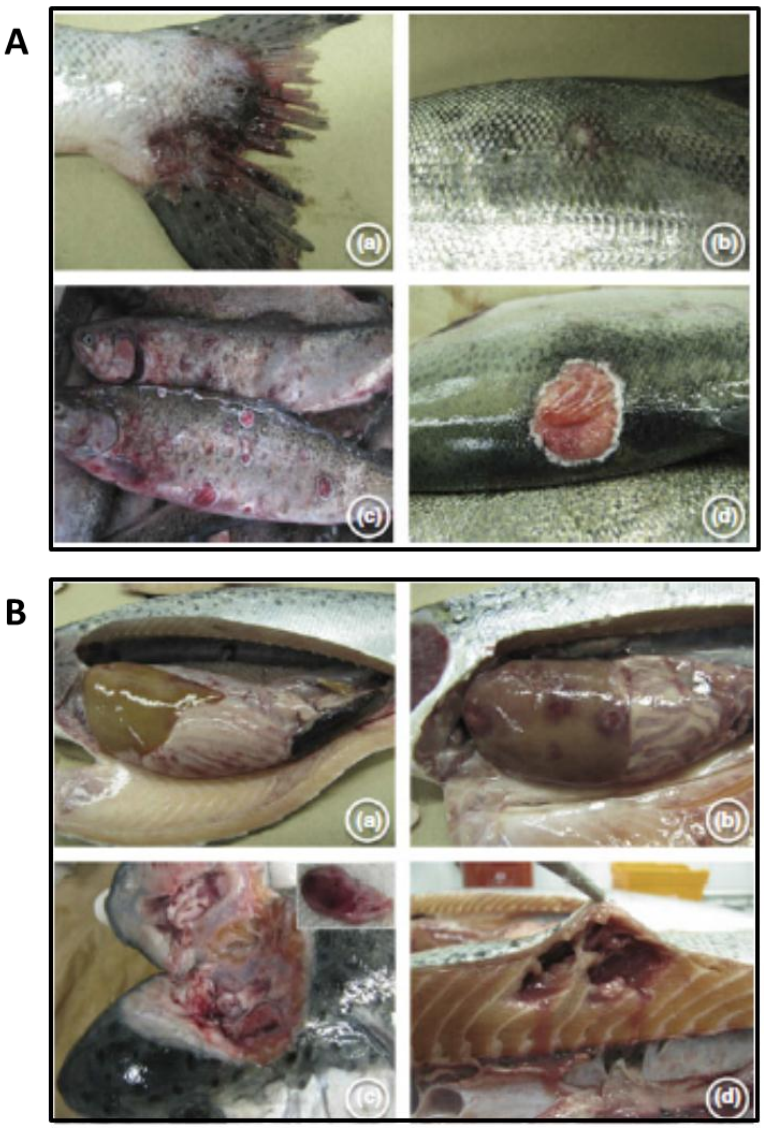

Figure 3: external (A) and internal (B) clinical signs of Piscirickettsiosis disease affecting salmonid species. (Rozas and Enriquez, 2014). In panel **A** it's observed petechial and ecchymotic hemorrhages in fins (a), raised scales (b), multiple and diffuse hemorrhagic skin ulcers (c) and focal skin ulcers. In panel **B** it is observed serosanguinous ascites, splenomegaly, pale liver and diffuse petechial hemorrhages (a – b), hyperaemia and diffuse hemorrhages and pethechiae in the encephalon (c) and caverns inside the skeletal muscle (d).

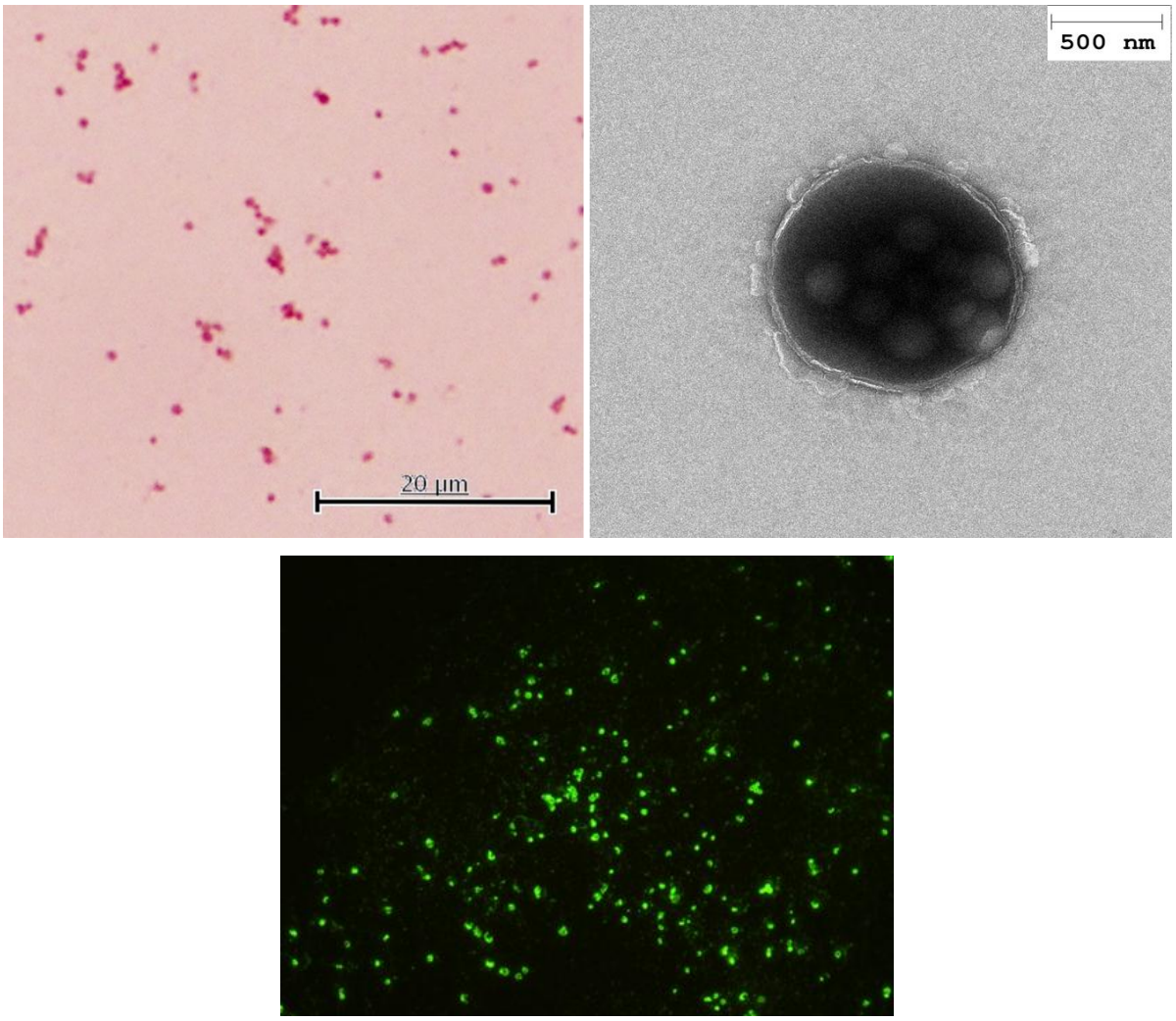

Figure 4: Gram staining, Transmission Electron Microscopy and IFAT images of *Piscirickettsia salmonis*.

The lack of effective treatments to control Piscirickettsiosis has emphasized the need to develop techniques for diseases prevention. Management of the disease is based on several husbandry practices including the application of immunostimulants of unproven efficacy and the control of vertical transmission by an expensive selection procedure during reproduction (Wilhelm *et al.*, 2006). Although vaccines made of inactivated bacteria have been successfully used to control certain bacterial disease in fish (Gudding *et al.*, 1999); preparations based on *P. salmonis* bacteria have not yielded significant protection against Piscirickettsiosis (Smith *et al.*, 1997; Kuzyk *et al.*, 2001). *P. salmonis* infections are septicemic and initially target monocytes followed by infection of the endothelium of the kidney, liver, spleen, heart, brain, intestine, ovary and gills of salmonids (Cvitanich *et al.*, 1991). The mechanisms used by this bacterium to survive and replicate inside the host cells are still poorly understood.

1.3 PATHOGENESIS AND VIRULENCE FACTORS

Phagocytosis is responsible for the internalization of microorganisms, damaged cells, and inert particles. Some intracellular pathogens inhabit vacuoles that interact with compartments of the biosynthetic pathway, while others escape from the phagocytosis or remain in vacuoles which neither acidify nor fuse with lysosomes (Berón *et al.*, 2002). The acid environment appears to be essential for *Coxiella burnetti* replication since raising the lysosomal pH with lysosomotropic amines or proton pump V-ATPase inhibitors reduces the growth of *C. burnetti* (Hackstadt and Williams, 1981; Heinzen *et al.*, 1996). Howe and Mallavia (2000) have shown, in an *in vitro* assay, increased protein synthesis in *Coxiella burnetti* incubated at pH 5.5 (endosomal pH) compared to that observed at pH 4.5 (lysosomal pH). Since *C. burnetti* replicates only when it is phagocytosed and transported to the phagolysosome, dissecting this transport pathway at the molecular level will help us to find a therapeutic approach for this infectious agent (Berón *et al.*, 2002). Many bacterial pathogens use Secretion Systems as part of their infection machinery to establish the infection inside the host cells. Protein secretion across membranes is a key aspect in bacterial virulence and occurs through a variety of mechanisms, from a simple one-component system to complex multi-component machineries.

The two main mechanisms used by pathogens to delivery substrates to the extracellular milieu or cytoplasm, are known as direct and indirect pathways. The indirect pathway is used for toxins without cell contact and the direct pathway for effector proteins that is cell contact dependent. Both have similar function which is to manipulate the environment so the bacteria can establish the infection and replicate. The macromolecules delivered are referred to as effectors because they affect and alter host cellular processes, resulting in the spread of the disease (Backert and Meyer, 2006). All these molecules described in pathogenicity from bacterial infection are denominated as virulence factor because the bacteria use them to invade the organism/host and continuing their life cycle.

1.3.1 PROTEIN SECRETION SYSTEMS

Currently there are several protein Secretion Systems described in prokaryotes. The secretion of the *E. coli* hemolysin is an excellent model of Type 1 Secretion System (T1SS). T1SS is comprised of three components: HlyB, HlyD and TolC. HlyB is an ABC transporter in the inner membrane which is a homodimer; HlyD is a trimeric inner membrane protein with a large periplasmic domain which contacts the outer membrane component TolC in the presence of the substrate HlyA. The Type 2 Secretion System (T2SS) also known as the General Secretion Pathway (GSP) is the major secretion pathway in Gram-negative bacteria. The best studied T2SS is from *Klebsiella oxytoca*. The components of T2SS are named Gsp to unify names between bacteria. This kind of Secretion System works together with the Sec pathway to export proteins outside the cell. In the T2SS, the secreted proteins are folded in the periplasm prior to secretion (Dalbey and Kuhn, 2012). To this T2SS belongs the Sec pathway, the *sec* genes named *sec*A, B, D, E, F, G and Y, which are involved in the export of proteins across the *E. coli* inner membrane. This Sec-dependent pathway works with two complexes inserted in the inner membrane, SecYEG and SecDF-YajC. It's proposed that the SecYEG translocon is the main complex working in this type of secretion, exporting the substrates, and the SecDF-YajC complex helps with the insertion and de-insertion of the SecA protein (cycling) to maintain the Proton-Motive Force (PMF) (Duong and Wickner, 1997). It's

described also that this Sec-dependent pathway can work together with the Type 1, 4 and 5 Secretion Systems, depending on the environmental conditions (Dalbey and Kuhn, 2012).

One of the most studied systems is the Type 3 Secretion System (T3SS) used by bacterial pathogens such as *Salmonella enterica* and *Shigella flexneri*, (Cornelis and Van Gijsegem, 2000). This Type 3 Secretion System (T3SS) is found in pathogenic and symbiotic bacteria that infect both animal and plant cells. The substrates from T3SS are named effector proteins and they are exported out of the cell in a 1-step process. This Secretion System involves more than 25 proteins that are highly conserved among the pathogenic bacterial species and several show sequence similarities with flagellar assembly genes. The Type 4 Secretion System (T4SS) is observed in both Gram-negative and Gram-positive bacteria. The evolutionary origin of the system is the conjugational system and it is employed for the transport of virulence proteins and DNA into eukaryotic cells, as well as for the conjugative transfer of plasmids from one bacterium to another. The T4SS of *Agrobacterium tumefaciens* is typical for this system and is employed to transfer a specific part of the tumor-inducing Ti-plasmid into a plant cell. This is called VirB/D System or also named Type 4A Secretion System (Dalbey and Kuhn, 2012). There also exists another kind of T4SS studied in *Legionella pneumophila*, named Dot/Icm or Type 4B Secretion System (Vogel, *et al.*, 1998; Segal, *et al.*, 1998). The Type 5 Secretion System (T5SS) includes autotransporters and 2-partner systems; one of the partners is the Sec-dependent pathway (Dalbey and Kuhn, 2012). The Type 6 Secretion System (T6SS) exists in most Proteobacteria that come into close contact with eukaryotic cells. The genetic clusters, which contain as few as 12 to more than 20 genes, are usually found in pathogenicity islands, and the gene expression is upregulated on contact with the host cell (Mougous *et al.*, 2007). It is possible that the T6SS has evolved from a bacteriophage and allows bacteria to use the tip of the T6SS needle to deliver the respective T6SS proteins with enzymatic activity into the host cell (Dalbey and Kuhn, 2012). The Type 7 Secretion System (T7SS), also termed ESX, is present in *Mycobacterium tuberculosis* which shows a highly unusual and complex cell envelope, so it uses specialized protein secretion system to secrete virulence factors. Mycobacterial genomes contain up to five genetic loci coding for T7SS, named ESX-1 to ESX-5 (Daleke, *et al.*, 2011). Finally, the Type 9 Secretion System or Por Secretion System involves the *porK, porL, porM,*

porN, porP, porQ, porT, porU, porV, porW and *sov* gene products (Sato, *et al.*, 2010; McBride and Zhu, 2013). Recently, it has been described that *Piscirickettsia salmonis* possess the Type 4B Secretion System which increased the gene expression levels of specific genes belonging to this T4BSS.

1.3.2 TYPE 4 SECRETION SYSTEM AND EFFECTOR PROTEINS

Many bacteria, such as *Agrobacterium tumefaciens*, *Helicobacter pylori*, *Bordetella pertussis*, *Brucella* sp., *Legionella pneumophila*, *Coxiella burnetii* and others, utilize functionally homologues systems known as Type 4 Secretion System (T4SS) for pathogenesis. T4SS are bacterial devices homologous to conjugation systems that are used by bacteria to deliver macromolecules (nucleoprotein complexes and proteins) across kingdom barriers (Christie and Vogel, 2000). The T4SS was divided into two subgroups: systems that resemble the *A. tumefacines* Vir system and the pKM101 Tra system were named Type 4A, and systems that resemble the *L. pneumophila* Icm/Dot System and the IncI plasmids Tra/Trb System were named Type 4B (Christie and Vogel, 2000). Up to date, two bacterial pathogens, *L. pneumophila* and *C. burnetii*, were shown containing a type 4B Secretion System (fig. 5), which was shown to be essential for *L. pneumophila* pathogenesis (Vogel, *et al.*, 1998), and recent information suggests that this is also the case for *C. burnetii* (Zamboni, *et al.*, 2003).

Gram-negative *L. pneumophila* is an environmental pathogen which normally replicates within a protozoan host. This intracellular facultative bacterium is an opportunistic human pathogen, and it causes the Legionnaires' disease. The disease is associated with efficient replication of *L. pneumophila* inside monocytes and alveolar macrophages with the latter being the primary site of bacterial growth. Internalized *L. pneumophila* forms a distinct *Legionella*-containing vacuole (LCV). Bacterial pathogens use a broad range of effectors to subvert and control normal cellular functions. Effectors are usually specialized proteins that are injected directly into the cytosol of the host cell by Type 3 Secretion System (T3SS) or a Type 4 Secretion System (T4SS). These secretion systems consist of a structurally conserved proteinaceous apparatus that is shaped like a needle (Galan and Wolf-Hatz, 2006).

Most of our current knowledge on this intracellular survival strategy comes from research on *Legionella pneumophila*. A functional T4BSS, encoded by a group of 24 genes called *dot/icm* (fig. 5), is essential for the protein secretion process (Segal, *et al.*, 1998; Vogel, *et al.*, 1998).

The distribution of these proteins from outer space to cytoplasm are present in a different manner among bacteria. Studies made in *Legionella* and *Coxiella* give us the possible orientation and localization of all these proteins and several protein complexes were identified. One of them is formed by DotH, DotG, DotF, DotC and DotD that joint the inner and outer membrane and is the functionally core complex. Another complex is formed by DotL, DotM and DotN that work as an ATPase for the secretion system. Also were found a few proteins in the periplasmic space like IcmX (free in periplasmic space) and DotK (inserted in outer membrane, periplasmic side); there are several proteins inserted in the inner membrane like DotU, IcmF, DotJ, DotV, DotE, IcmT, DotI, IcmV, DotP and DotA (ATPase), and some cytoplasmic proteins also like DotB, that work as ATPase. Also was found the chaperone subcomplexe IcmS/IcmW (fig. 5) (van Schaik *et al.*, 2013).

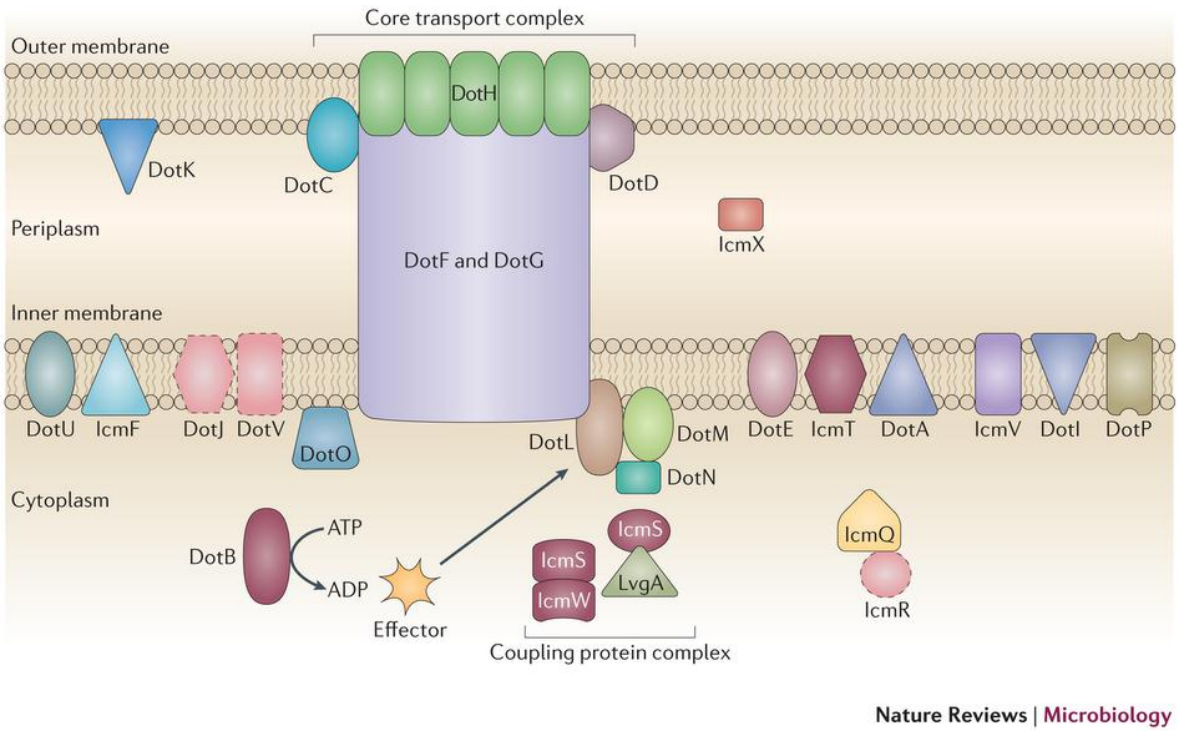

Figure 5: proposed model for distribution and localization of T4BSS proteins in *Legionella pneumophila* and *Coxiella burnetii* (van Schaik, *et al.*, 2013).

Transport of these effector molecules results in the biochemical manipulation of the host cell processes, promoting a favorable replicative niche for the bacterium and its survival. Pathogens such as *L. pneumophila* contain T4SS genes (*dot/icm*) that are associated with the establishment of a replication-permissive phagosome in eukaryotic hosts after phagocytosis (Chien *et al.*, 2004).

Although regulated assembly of these specialized Secretion Systems is often coordinated tightly with the expression of their cognate secretion substrates, common recognition elements must exist to not only promote transport by the correct pathway, but also to serve as a control mechanism for choosing only a particular set of proteins for transport among a pool of cytoplasmic candidates (Cambronne and Roy, 2006). First identified from genes called "defect organelle trafficking" (*dot*) and "intracellular multiplication" (*icm*) that were required for replication of the pathogen *L. pneumophila* in eukaryotic host cells, the Type 4B transport system now has been identified also in *Coxiella* and *Xanthomonas* species (Seshadri, *et al.*, 2003; Thieme, *et al.*, 2005).

Many Dot/Icm substrate proteins have homology to eukaryotic proteins, and the sequencing of several *L. pneumophila* strains has revealed extensive genomic plasticity which affects the number of copies and the diversity of Dot/Icm substrates found in the genome (Cazalet *et al.*, 2004). T3SS substrate recognition is mediated by signals located in the N-termini of effector proteins, whereas the T4SS recognizes an element in the C-termini (Cambronne and Roy, 2006). *Legionella pneumophila* and *Coxiella burnetii* are evolutionary closely related bacteria that belong to the Gamma-proteobacteria (Weisburg *et al.*, 1989).

Gómez *et al.* (2013) described the presence of *dotG* and *dotH* genes during infectious process induced by *Piscirickettsia salmonis* on CHSE-214 cell line after 48 hours post infection. Recently, Isla *et al.* (2014) demonstrated the presence of the *clpB* and *bipA* genes at transcript levels in an infection induced by *Piscirickettsia salmonis* in the SHK-1 cell line. Those genes are important during infectious process as effector proteins with unknown function in *Piscirickettsia salmonis*.

Currently, our knowledge about this bacterium is limited to a few articles that describe the induction of apoptosis in phagocytic host cells (Rojas, *et al.*, 2010), the presence of the type 4B secretion system at transcript level (Gómez, *et al.*, 2013), the possible evasion of the phagocytic

pathway after internalization in the host cell (Gómez, *et al.*, 2013), the increase of gene expression levels of two virulence factors with a key role in the intracellular survival (ClpB and BipA) (Isla A, *et al.*, 2014), and recently the complete genome for the LF-89 strain (Pulgar *et al.*, 2015). With the exception of the information mentioned above, all other knowledge comes from the study of the related pathogens *L. pneumophila* and *C. burnetii*, both Gammaproteobacteria like *P. salmonis*.

1.4 HYPOTHESIS AND OBJECTIVES

Using all this information above and some preliminary results I propose the **Hypothesis:**

"The effector protein SdhA and Secretion System Type 4B (Dot/Icm) from *Piscirickettsia salmonis* are involved in the bacterial pathogenicity during *in vitro* infection"

General Objective:

- To Assess the expression and function of *dotG, dotH* and *sdhA* genes from Secretion System type 4B (Dot/Icm) in *Piscirickettsia salmonis* during *in vitro* infection.

Specific Objectives

- To determine the mRNA levels of *dotG, dotH* and *sdhA* genes from Secretion System type 4B in *P. salmonis* during *in vitro* infection.
- To analyze the presence at protein levels of DotG, DotH and SdhA from Secretion System type 4b during *in vitro* infection induced by *P. salmonis*.
- To determine the role of SdhA during *in vitro* infection by blocking its translation with Antisense Oligonucleotide (ASO).

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICAL REAGENTS

The following reagents were used during the research work:

Amresco: N,N,N´,N´-tetrametiletilendiamine (TEMED), Sodium Bicarbonate ($NaHCO_3$).
AppliChem: acrylamide/bis-acrylamide (N,N´-metilenbisacrilamide).
Biological Industries: bovine serum albumin (BSA), trypsin-EDTA.
CalbioChem: glycine, glycerol.
Fermentas Life Sciences: pageruler plus prestained protein ladder.
Gibco: fetal bovine serum (FBS), Leibovitz´s medium L-15.
Hyclone (ThermoScientific): antibiotic solution (10 U/mL penicillin G, 10 μg/mL streptomycin, 25 μg/mL amphotericin B), goat anti-chicken HRP conjugated antibody, Pierce ECL Western Blotting Substrate.
IDTDNA Technologies: oligonucleotides for molecular biology (Real Time RT-PCR), oligo dT (15- 27bp).
Maestrogen: accuruler RGB prestained protein ladder.
Merck: hydrochloric acid (HCl); glacial acetic acid (CH_3COOH); ethanol (CH_3CH_2OH), sodium hydroxide (NaOH); tris (hydroxymethyl) aminomethano ($H_2NC(CH_2OH)_3$); potassium chloride (KCl); Triton X-100; Coomassie blue R-250 ($C_{45}H_{44}N_3NaO_7S_2$); ammonium persulfate ($(NH_4)_2S_20_8$); Tween®20; bromophenol blue; β-mercaptoethanol, sulfuric acid 95-97% (H_2SO_4).
Metabion: oligonucleotides for molecular biology (Real Time RT-PCR) and antisense oligonucleotides phosphorothioate (ASO).
Promega: Random Primers, M-MLV Reverse Transcriptase, RNasin.
SantaCruz Biotechnologies: EEA-1, Rab7, LAMP-1 antibodies.

Sigma Chemical Co: Tittermax gold adjuvant, Freund's adjuvant Complete and Incomplete.

Sigma-Aldrich: TMB 3,3,5,5-tetramethylbenzidine.

USBiological: sodium dihydrogen phosphate monohydrate ($Na_2HPO_4H_20$); sodium hydrogen phosphate (NaH_2PO_4); sodium dodecyl sulfate (SDS), anhydride monobasic potassium phosphate (KH_2PO_4).

Winkler: sodium chloride (NaCl); 2- propanol ($CH_3CH(OH)CH_3$), Nonidet P40 ($(C_2H_4O)nC_{14}H_{22}O$), lab grade Ethanol, lab grade Methanol, 2-Propanol, Chloroform, Ether.

2.1.2 BUFFERS AND SOLUTIONS

PBS 1X: 100 mL of PBS 10X (NaCl 0.138M; KCl 0.0027M; Na_2HPO_4 0.01M, pH 7.4) and fill with distilled water to 1L.

TBS 1X: 100 mL of TBS 10X (NaCl 0.15M; Tris 0.01M pH 8.0) and fill with distilled water to 1L.

TAE 1X: 20 mL of TAE 50X (2M Tris, 50mM EDTA pH 8.0) and fill with distilled water to 1L.

RIPA buffer: Tris-HCl 50mM; NaCl 150mM; NP-40 1%; sodium deoxycholate 0.5%; SDS 1% at pH 7.5.

Upper buffer: Tris-HCl 0.5M, pH 6.8.

Lower buffer Tris-HCl 1.5M, pH 8.8.

Loading buffer: Tris-HCl 62.5 mM; SDS 2%; glycerol 10%; β-mercaptoethanol 10%; bromophenol blue 0.01% a pH 6.8.

Running buffer: Tris 0.1M; glycine 0.768M, SDS 0.4%.

Blotting buffer: Tris 25M, glycine 193mM, methanol 20%.

Tris-HCl buffer: Tris 0.0625M at pH 6.8.

Western blot blocking solution: powder milk 10% (p/v) in PBS 1X.

Fix solution SDS-PAGE: 2-propanol 25%, acetic acid 10%.

Staining SDS-PAGE: Coomasie blue R-250 0.3%; methanol 50%; acetic acid 10%.

De-staining solution SDS-PAGE: acetic acid 7%, methanol 30%.

Silver staining solution: Fix buffer (50% Methanol; 10% Acetic Acid); Wash buffer 1 (50% Ethanol); Wash buffer 2 (10% Ethanol); Blocking buffer (0.2% (w/v) $Na_2S_2O_3$ in H_2O); Staining buffer (0.1% $AgNO_3$, Formaldehyde stock); Develop buffer (6% (w/v) Na_2CO_3, Formaldehyde stock); Stop buffer (10% Acetic Acid)

Washing solution western blot: Tween-20 0.1% (v/v) in TBS 1X.

Antibody solution: NaCl 0.25M 14.61g/L; NaH_2PO_4 2.9mM 0.4g/L; Na_2HPO_4 7.1mM 1.008 g/L; BSA 1500 mg/L a pH 7.6.

Dialysis solution: NaCl 0.25M 14.61g/L; NaH_2PO_4 2.9mM 0,4g/L; Na_2HPO_4 7.1mM 1.008 g/L, pH 7.6.

2.1.3 EQUIPMENTS

Stirring magnetic Nuova (Stirrer), orbital stirring Polymax 1040 (Heidolph), analytic weight AS310/C/2 (Radwag), *Balanza granataria*, WTB 3000 (Radwag), thermoregulatory bath (Memmert), laminar flow chamber NU425-400E (Nuaire), centrifuge model 5702 (Eppendorf), refrigerator centrifuge high speed CR 22GIII-R20A2 (Hitachi), power supply Power Pac HC (Bio-Rad), Biosafety Cabinet Class 2 (Esco), homogenizer Silent Crusher M (Heidolph), orbital shaker incubator LM-510-2/5 (mcr), incubator FOC 2251 (VELP Scientifica), dialysis membrane Spectra/Por MWCO: 25,000 (Spectrum), Polyvinylidene difluoride membrane (PVDF) (ThermoScientific), Micropipettes P2, P10, P20, P200, P1000, P5000 (Axygen), optical microscope DM 2500 (Leica), inverted microscope CKX41 (Olympus), pH-meter Orion 3 Star Plus pH Benchtop Meter (ThermoScientific), electrophoresis system Mini-Protean Tetra System (Bio-Rad), blotting system Mini trans-blot cell (Bio-Rad), Sonicator μltrasonic Liquid Processors XL-2000 series (Misonix), Vortex Mixer VM-2000 (mcr), Eppendorf Mμltiporator (Bacteria Modμl 4308 805.005).

2.1.4 ANIMAL MODEL

Hens *Lohmann* strain: Adult hens of about 6-8 weeks of age were used, which were provided by the nursery of the Institute of Animal Pathology, Faculty of Veterinary Sciences, Universidad Austral de

Chile and were kept under daily regime of 12h light/dark with a water-based food (commercial pellet) *"ad libitum"* according to current regulations of biosecurity measures from "Manual of Standards for Biosafety" CONICYT 1994. Once the hens were immunized diet remained the same way until the end of the collection of eggs.

Rats Sprague Dawley: young rats since 1 month of life (100g of weight) were kept under controlled condition by the authorized personnel. When the rats had around 150g of weight they were inoculated with at least three injections of synthetic peptides during 6 weeks in total, with a waiting time between injections of two weeks. At the end of the inoculation, all the blood from all the rats was collected and the serum extracted to get the polyclonal antibodies for the subsequent experiments.

2.1.5 CELL LINE AND BACTERIAL STRAIN

SHK-1: adherent cell line from kidney head of Atlantic salmon. To maintain the cell line, Leibovitz's medium L-15 supplied with 10% of FBS and 1X of antibiotic mix was used. The cells were grown in T25 or T75 flask at 18°C without CO_2 and with periodic medium changes every 5 days. When the cells were confluent, cells were detached from the bottom using 3 ml of trypsin with PBS 1X (1:1), centrifuged at 2000xg for 5 minutes and the cells resuspended from the pellet in fresh L-15 medium with FBS and antibiotic. The day before initiate the infection experiments, the cells were washed three times with PBS 1X and added to L-15 medium with 2% of FBS without antibiotic and kept in this conditions during the whole experiment.

AUSTRAL-005: this strain was isolated from Rainbow trout diagnosed with Piscirickettsiosis. It was kindly provided by BIOVAC S.A. This bacterium was used for the growing assays in medium AUSTRAL-SRS broth, protein extracts, RNA extracts and for the infection experiments in eukaryotic cells.

LF-89: this strain ATCC VR-1361 was used to compare the cytopathic effect on eukaryotic cells.

All the infection experiments were carried out using MOI 10, with incubation times termed short times (0h, 2h, 4h, 8h, 16h, 24h) and long times (2d, 4d, 6d, 8d).

2.2. METHODS

2.2.1 BACTERIAL CULTURE IN LIQUID MEDIUM

The bacterium *P. salmonis* was cultured in liquid medium *AUSTRAL-SRS Broth* (Yáñez et al., 2012). This medium is based on marine medium, supplied with peptone and yeast extract as nitrogen source. Furthermore, it is supplied with FBS, salts, aminoacid and glucose. Doing measurements of absorbance at 600nm, it is possible to determine the exponential growing stage in culture from the third day reaching the stationary stage at sixth day of culture. To perform the experiments, the bacteria were always cultured at 18°C with 150 rpm of shaking during five days. After this time, to confirm the purity of the culture, the Gram staining was performed. Also the PCR analysis with specific primers was done to confirm the purity of the bacterial culture. Once the exponential stage was reached, the bacteria were collected by centrifugation at 3500xg to proceed with the experiments or protein extract.

2.2.2 *Piscirickettsia salmonis* GRAM STAINING

Approximately 10µL of bacteria growing in liquid medium *AUSTRAL-SRS* Broth, were put onto a slide. After dried, sample was covered first with the primary reactive (crystal violet) for one minute. After that, the sample was washed with distilled water and then the second reactive was used (lugol) and again incubated for one minute. Then, the slide was washed again with distilled water. Now, it was time for the third reactive (acetone/alcohol) to decolorize the sample for a few seconds (3-10 seconds). Finally, the last reactive (safranin) was used to counterstain the sample during 30-60 seconds. After this time, the slide was washed with distilled water, the sample dried and the staining reaction checked under the optical microscope using immersion oil (100X).

2.2.3 PCR AND REAL TIME PCR

From each infection experiment, RNA was extracted by using 1000µL of Trizol Reagent (Ambion) for each sample and then mixed with 200µL Chloroform solution, vortexed for 15 seconds and centrifuged

20 minutes at 12000xg at 4°C. The upper soluble supernatant was recovered in a new tube (near 300µL) and mixed with 500µL of 2-Propanol and incubated at -20°C overnight. Next day, the total RNA extraction was carried out by commercial (Axygen) Total RNA extraction kit. The nucleic acid was quantified with Nanodrop Maestrogen Equipment. To ensure no contamination with DNA, 5µg of total RNA from all samples were treated with DNase Epicenter at final volume of 50µL incubated at 37°C for 10 minutes. For the cDNA 1µg of total RNA from each sample was used and the Reverse Transcription (M-MLV Promega) reaction was carried out by manufacturer's protocol. Briefly, 1µg of RNA from each sample at first step was mixed with 1nM of Random Primers and oligo dT mix (oligo dT from 15 to 27 bp) and incubated at 70°C for 5 minutes. After this step, the RNA/primer was mixed with the secondary solution (5µL RT buffer 5X, 1.25µL dNTPs 10mM, 0.75µL RNaseOUT 40U/µL) and adjusted with Nuclease Free water to 25µL of final volume. The RNA mixed solution was incubated 60 minutes at 37°C. The PCR and Real Time PCR were carried out with the same thermal profile as follows: initial denaturation at 95°C for 5 minutes; denaturation in the cycle at 95°C for 15 seconds; annealing at 60°C for 15 seconds; extension in the cycle at 72°C for 15 seconds; final extension at 72°C for 5 minutes. For Real Time PCR, after the extension step in the cycle, the melting curve from 55°C to 95°C was determined to evaluate the specificity of the primers. For PCR 35 cycles and for Real Time PCR 40 cycles were used. For all the conventional PCR reaction GoTaq Flexi 2X (Promega) and for Real Time PCR SYBR-Green 2X kit (Axygen) were used. 1µL of cDNA from each sample was used in a conventional PCR machine for Real Time PCRs. The primers used for each detection are described in table 1. As normalizing gene 23S, from *P. salmonis* strain AUSTRAL-005 was used and as a calibrator for Relative Quantification, it was used the 0 hour post infection in all infection experiments. To confirm the purity of the bacterial culture, the same 23S primers proven with samples from *E. coli* and *Francisella noatunensis* showing not amplification signal, were used.

2.2.4 BCA PROTEIN QUANTIFICATION

To get the total protein extract from the bacteria, the bacterial pellet was collected by centrifugation of the liquid medium *AUSTRAL-SRS* broth; after resuspension in 500µL RIPA buffer (Tris-HCl 50mM; NaCl 150mM; NP-40 1%; sodium deoxycholate 0,5%; SDS 1%; pH 7,5) with PMSF 1X, and incubation on ice for 20 minutes, cells were sonicated at 80 Watts for 2 minutes with pulses every 20 seconds. Centrifugation at 16000xg for 20 minutes at 4°C collected the proteins in the supernatant. The BCA protein quantification method is a colorimetric way based on the bicinchoninic acid (BCA). This

method is based on the reduction of copper 2+ to copper 1+ by the protein sample in alkaline medium (Biuret reaction) and uses the precise colorimetric and sensitive detection of the copper 1+ by bicinchoninic acid. The product of this reaction is purple by the reaction of two molecules of BCA with one molecule of copper 1+. The water soluble complex can be measured at 562nm, increasing in a linear fashion according to the protein amount in the range from 20 to 2000 μg/ml. To quantify the protein extract from *P. salmonis* during infectious process the commercial kit Pierce™ BCA Protein Assay Kit (Pierce) was used. To quantify the samples it is necessary to prepare a standard curve with several dilutions in the range from 25 to 2000μg of albumin from BSA stock. This assay was performed in a 96 well plate adding 200μL of BCA reactive plus 25μL of each diluted 1:25 samples (sample: water) or each standard curve point. Then the plate was incubated at 37°C for 30 minutes. After the incubation time the plate was read at 562nm and the concentration of the sample was measured according to the standard curve. All the samples were analyzed twice.

2.2.5 HENS/RATS INOCULATION WITH SYNTHETIC PEPTIDES

Three hens and six rats were immunized with synthetic peptides, each with specific peptide according to analysis from the putative DotH, DotG and SdhA proteins from *P. salmonis* (table 2). The conjugation of the synthetic peptide was made as follows: the synthetic peptide was resuspended in Sodium Borate buffer pH 9 at 2mg in 225μL. This 225μL (8μg/μL) of resuspended peptide were conjugated with 6.7μL of Hemocyanin blue carrier (BioSonda S.A.)(1mg from stock 150mg/mL), 77μL of glutaraldehyde 2.5% in sodium borate buffer pH 9. This mixed solution was stirred overnight at room temperature in Eppendorf tubes. The next day the solutions were dialyzed against 0.15M NaCl in dialysis membranes with an 8 kDa cut off with shaking at 4°C. After this step the conjugated peptide (5μg/μL) was recovered in a new Eppendorf tube and stored at 4°C until use.

For each animal according to its weight a first injection of 200-300μg (rats and hens respectively), and 100 and 200μg for the subsequent injections were used. The emulsion was prepared as follows: the conjugated peptide was used and mixed with Titermax Gold Adjuvant in equal proportion as the conjugated amount used (v/v). All the mixes were prepared with PBS 1X to obtain the right volume to inoculate at least 5 injection sites in the back (sub-cutaneous) of the rats and in the breast of the hens (intramuscular). The pre immune sera and the pre immunized eggs were collected before the first inoculation as a control for the antibody production. After 2 months the sera from rat bloods were collected by centrifugation at 3500xg at 4°C. The eggs collection was performed using the protocol

described by Akita and Nakai (1992), and modified in our laboratory. The eggs were collected after the first immunization and the IgY purification was carried out from a "pool" from these eggs. Briefly, the yolk was separated from the white part and softly washed with distilled water over a smooth paper. Then, the yolk was punctured with a sterile syringe needle and transferred into a test tube; it was diluted 10 times with sterile water and the pH was changed to 5.2 with 0.1N HCl. This solution was incubated at 4°C overnight without shaking to induce the formation of the lipids microspheres. Next day this emulsion was filtered with Whatman paper and funnel to separate the lipids from the clear solution. The proteins from the filtered solution were precipitated using 35% of ammonium sulfate for 30 minutes with shaking at 4°C. Then, the suspension was centrifuged for 25 minutes at 10000xg at 4°C. The supernatant was recovered in a test tube and it was precipitated again now with 30% of ammonium sulfate to reach a final concentration of 65% and incubated for 30 minutes with shaking at 4°C. Then the mix was centrifuged at 10000xg for 25 minutes at 4°C. After that, the recovered pellet was resuspended in antibody buffer BSA 1X pH 7.6 (NaCl 0.25M 14,61 g/L; NaH2PO4 2.9mM 0,4g/L; Na_2HPO_4 7.1 mM 1,008g/L; BSA 15mM 1500mg/L) for each yolk and the solution was dialyzed against 2L antibody buffer without BSA using dialysis membrane with 12kDa cut-off. Finally, both polyclonal antibodies were stored separately in aliquots at -20°C until use.

2.2.6 SDS-PAGE ANALYSIS

To perform polyacrylamide gel electrophoresis in denaturant condition, Mini protean III system from BioRad was used, following the protocol developed by Leammli (1970) in which the separator gel was prepared at a final concentration of 15% of Acrylamide/Bis-Acrylamide 29/1 in Tris-HCl buffer (1,5M; SDS 10% pH 8.8) and polymerized using 400µL of ammonium persulfate 10% and 16µL of TEMED 0.2% as catalyzer. The spacer gel was prepared with Acrylamide/Bis-Acrylamide 29%/1% at a final concentration of 4% in Tris-HCl buffer (0,5M; SDS 10% pH 6.8) and polymerized with 80µL of ammonium persulfate 10% and 8µL of TEMED as catalyzer. To characterize the protein pattern, 20µg from all samples of free cell liquid medium bacteria and from the infectious process induced by this bacteria, were separate in the SDS-Gels using a 5X loading buffer (Tris-HCl 62,5mM pH 6.8; SDS 2%; glycerol 10%; β-mercaptoethanol 10%; Bromophenol blue 0,01%). All the samples were denatured before the electrophoresis by heating at 95°C for five minutes. The running buffer (Tris 250mM, glycine 190mM, SDS 0,01%) was used for all the SDS-gels applying an electric field of 70 Volts for 2 and a half hours approximately. To visualize the protein pattern in the gels the Coomasie blue staining

(methanol 40%; acetic acid 10%; Coomasie blue R-250 0,1%) was used for 2 hours and then washed with destaining buffer (methanol 30%; acetic acid 7%). Finally, the gels were dried with a Dry Gel Buffer (22% ethanol, 2% glycerol, 1% 2-propanol, mixed in distilled water) to get the images with Fluorshot, using the equipment SC750, BIOSENS.

2.2.7 WESTERN BLOT ANALYSIS

For western blot analyses the electrophoretic samples described above were used. The proteins were blotted to a PVDF membrane, using the Bio-Rad blotting system (Trans-Blot SD), with blotting buffer in an electric field with 450 mA for 100 minutes at 4°C. Then, with the proteins in the PVDF membrane, the protein free spaces were blocked with blocking buffer (TBS 1X, 0,1% (v/v) Tween-20, 10% (p/v) powder milk) during one hour with shaking at room temperature. Then, the membranes were incubated with dilution of the first polyclonal antibody in different blocking buffer (TBS 1X, 0.1% (v/v) Tween-20, 3% (p/v) powder milk) overnight at 4°C. Thereafter, the membranes were washed with washing buffer (TBS 1X, 0.1% (v/v) Tween-20) three times ten minutes each with shaking, at room temperature. Then, the membranes were incubated with a dilution (1:2000) of the secondary antibody conjugated with horse-radish peroxidase (HRP) against the animal model used (rat or hen) in the same blocking buffer for the first antibody during at least two hours at room temperature with shaking. Thereafter, the membranes were washed again with washing buffer three times ten minutes each with shaking at room temperature. Finally, the interaction between antigen and antibody was detected with commercial kit ECL Pierce Western Blotting Substrate, based on a chemiluminescent reaction where the substrate is coupled with the HRP. The images were obtained with the G-BOX Chemi XT4, SYNGENE.

The polyclonal antibodies used in these western blot analyses were from the animal models (rat and hen) induced by synthetic peptides. The dilution for the primary antibody was standardized at 1:250 for each polyclonal antibody, and for the secondary antibody, the standard dilution was 1:2000. The sample amount used in SDS-PAGE was 20μg of total protein extract from infectious process induced by *P. salmonis* in cell line SHK-1.

2.2.8 ANTISENSE OLIGONUCLEOTIDES TECHNIQUE

Using the nucleotide sequence from the partial genome of the strain AUSTRAL-005 from *P. salmonis*, the Shine-Dalgarno sequence in the possible *dotH, dotG* and *sdhA* genes (table 2) was used to design the Antisense Oligonucleotides (ASO). The ASO were applied to cells by electroporation to inhibit translation of the respective genes. For electroporation assay, a 10% glycerol solution containing each ASO in a final concentration of 500nM was used. For these experiments, the bacteria were grown in AUSTRAL-SRS broth for 5 days (exponential growing phase). Then 1mL of bacteria was collected in separate 1.5mL Eppendorf tubes by centrifugation at 3500xg for ten minutes at 4°C. After that, the medium was replaced by a mix between 10% glycerol and 500nM of each ASO (100µL final volume mix). The electroporation experiment was carried out in Eppendorf Multiporator using 1000Volts and sterile cuvettes with caps. Once the bacteria were electroporated, this 100 µL mix was inoculated in AUSTRAL-SRS broth medium until the fifth day of growth to extract total proteins and carry out the western blot analyses with specific antibodies against DotH, DotG and SdhA proteins.

2.2.9 MULTIDIMENSIONAL PROTEIN IDENTIFICATION BY MASS SPECTROMETRY (MudPIT)

2.2.9.1 PROTEOLYSIS

The lyophilized proteins were resuspended in 6M guanidine hydrochloride, 25mM NH_4HCO_3, pH 7.5, and reduced with 2mM dithiotreitol (DTT) for 30 min at room temperature, and then alkylated by 10mM iodoacetamide for 30 min in darkness at room temperature. The reaction was diluted 7 times with 25mM NH_4HCO_3, pH 7.5 and 2µL of 0.1ng/mL of modified trypsin (Promega) was added. The trypsin reaction was incubated 16h at 37°C. The reaction was terminated by addition of acetic acid to pH 2.0.

2.2.9.2 MULTIDIMENSIONAL PROTEIN IDENTIFICATION BY MASS SPECTROMETRY (MudPIT)

All samples were concentrated on a centrivac concentrator (Labconco) to a final volume of 20µL and loaded on a 350µm ID fused silica 2D HPLC triphasic peptide trap column packed in-house with 3cm of a desalting C18 reversed phase (100Å, 5µm, Magic C18 particles, Michrom Bioresources), followed by 3cm of a strong cation exchange column (300Å, 5µm PolySULFOETHYL A, PolyLC inc) and finally with 3cm of resolving C18 reversed phase. The peptide trap was mounted on the loop of a nanlLC (ThermoFinnigan). Following a wash with 0.1% formic acid for 30 min at 0.5µL/min, the efflux of the peptide trap column was directed to a 10cm resolving reversed phase column (100Å, 5µm, Magic C18 particles, Michrom Bioresources), mounted on the electrospray stage of a FT IC mass spectrometer (LTQ FT, ThermoFinnigan). The peptides were separated on-line using 15 salt steps (0, 10, 30, 50, 100, 150, 200, 250, 300, 350, 400, 500, 1000, 1500, and 2000mM NH_4CH_3OO) followed by a 0 – 90% acetonitrile gradient in 120min at flow rate of 350nl/min. An electrospray voltage of 1.9kV was used, with the ion transfer temperature set to 250°C. The mass spectrometer was controlled by the Xcalibur software to perform continuously mass scan analysis on the FT followed by MSMS scans on the ion trap of the six most intense ions, with a dynamic exclusion of one repeat scans of the same ion, 30s repeat duration and 90s exclusion duration. Normalized collision energy for MS/MS was set to 35%.

2.2.9.3 DATA ANALYSIS

For data analysis all tandem mass spectra all MS/MS samples were analyzed using Sequest (Eng *et al.*, 1994), included in the proteome discoverer package (Thermo Fisher Scientific, San Jose, CA, USA; version 1.4.0.288) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Sequest was set up to search uniprot-Piscirickettsia+salmonis.fasta (9279 entries) assuming the digestion enzyme trypsin, and a maximum of 2 missed cleavages. X! Tandem was set up to search the same protein database

(also assuming trypsin). Sequest and X! Tandem were searched with a fragment ion mass tolerance of 0.80Da and a parent ion tolerance of 50PPM. Carbamidomethyl of cysteine was specified in Sequest and X! Tandem as a fixed modification. Deamidation of asparagine and glutamine and oxidation of methionine were specified in Sequest as variable modifications. Dehydrated of the n-terminus, glu->pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln->pyro-Glu of the n-terminus, deamidation of asparagine and glutamine and oxidation of methionine were specified in X! Tandem as variable modifications.

2.2.9.4 CRITERIA FOR PROTEIN IDENTIFICATION

Scaffold (version Scaffold_4.4.1.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 72.0% probability to achieve an FDR less than 0.5%. Peptide Probabilities from X! Tandem were assigned by the Peptide Prophet algorithm (Keller *et al.,* 2002) with Scaffold delta-mass correction. Peptide Probabilities from Sequest were assigned by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al.*, 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins were annotated with GO terms from NCBI (downloaded Mar 26, 2015) (Ashburner *et al.*, 2000).

2.2.10 STATISTICAL ANALYSIS

All the experiments were done in triplicate showing the average data plus standard deviation (Analysis of Variance, ANOVA, Sokal y Rohlf, 1980). The significances between the results and controls were established using t Student's test and it was used the P value significance of $P<0.05$.

3. RESULTS

Piscirickettsiosis is caused by *Piscirickettsia salmonis*, a Gram-negative facultative intracellular pathogen, that infects salmonids fish species inducing highly mortality and economic losses in salmon farming industry in Chile. This pathogen has the ability to replicate inside the infected cell (Gómez *et al.*, 2013). Currently, there is only limited knowledge about how this bacterium can establish the infection in macrophages, using the GSP Sec-dependent pathway and type 4B secretion system (T4BSS) to translocate effector proteins that subvert the infected cell to its own benefits (Gómez *et al.*, 2013). To evaluate this task, specific genes belonging to Sec-dependent pathway and T4BSS were looked for in the partial genome of AUSTRAL-005 strain available in our laboratory.

3.1 BIOINFORMATIC ANALYSIS

As a first step, genes belonging to the GSP Sec-dependent pathway and T4BSS were looked for in NCBI database, described for several pathogens, focused in *Legionella penumophilla* and *Coxiella burnetii*. After that, conserved domains present in each multiple alignment were looked for in the partial genome of *Piscirickettsia salmonis* AUSTRAL-005 strain. The possible genes found were *secY, secE, secG, secD, secF, yajC* and *yidC* for Sec-dependent pathway, several *icm/dot* genes for T4BSS and *sdhA* gene described as an effector protein to T4SS (figure 6). Genes from Sec-dependent pathway were found in different contigs, however, the proteins that form the secondary holotranslocon SecDF/YajC were found in the same contig in the same strand. The gene for the auxiliary protein YidC from Sec-dependent pathway was found in a different contig; genes from T4BSS were all found grouped in the same contig separately each other in three copies in the bacterial genome. The T4SS described effector protein SdhA was found alone in a different contig.

Once, all the possible genes for the Sec-dependent pathway, T4BSS and effector protein were found in the genome, a multiple alignment was made to compare the derived aminoacid sequences of *sdhA* genes from *L. pneumophila* and *P. salmonis* among other sequences present in

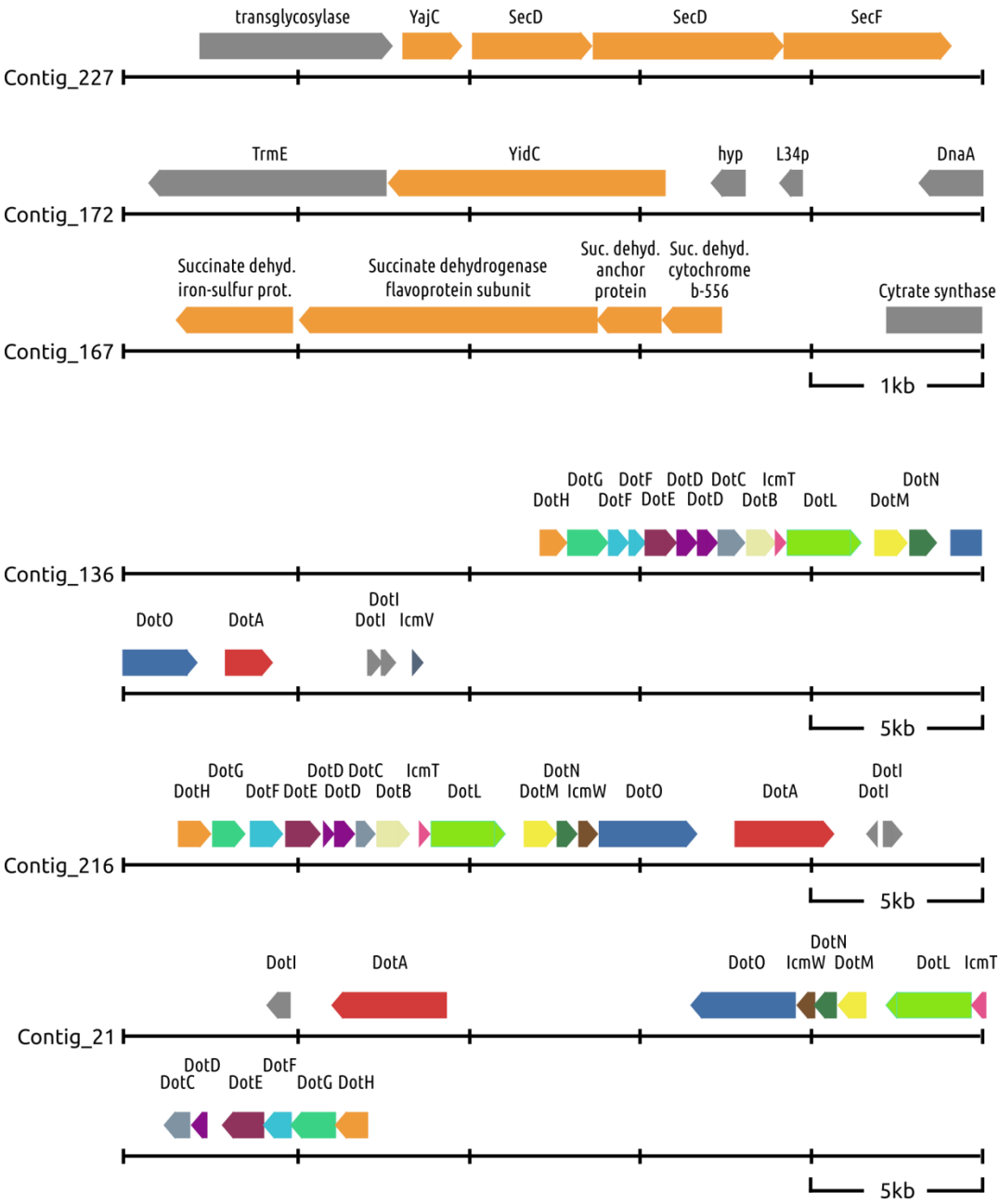

Figure 6: Bioinformatic analyzes of partial genome from *Piscirickettsia salmonis* AUSTRAL-005 strain to predict the putative location and genes of GSP (Sec-dependent) Contigs 227 and 172, T4BSS (Dot/Icm) Contigs 136, 216 and 21, and the putative effector protein SdhA Contig 167.

NCBI database (figure 7). The comparison revealed the similarities among all these proteins described in the databases even the possible SdhA protein from *P. salmonis*.

3.2 SPECIFIC GENE DETECTION

Using the information obtained above, specific primers were designed to amplify the *secD, secF, yajC, yidC, dotG, dotF, dotH* and *sdhA* genes from RNA extracted during an *in vitro* infection on SHK-1 cell line after short times (0h, 2h, 8h, 16h, 24h) and long times (2d, 4d, 6d) induced by the bacteria (figure 8). For standardization of the primers gradient conventional PCR was performed using 57°C, 60°C, and 62°C to check specific amplification of the PCR products. According to the results, 60°C for all the qRT-PCR experiments was chosen as annealing temperature (table 1).

A minimal amount of two primers for each candidate gene were used and checked for PCR amplification choosing the better primer pair from each gene. As a normalizer gene 23S rRNA specifically designed from *P. salmonis* AUSTRAL-005 strain was used. To check also the specificity of the primers, DNA and RNA from other bacteria like *E. coli* and *Francisella noatunensis* was used, where amplification wasn't observed at the same conditions used to the previous qRT-PCR.

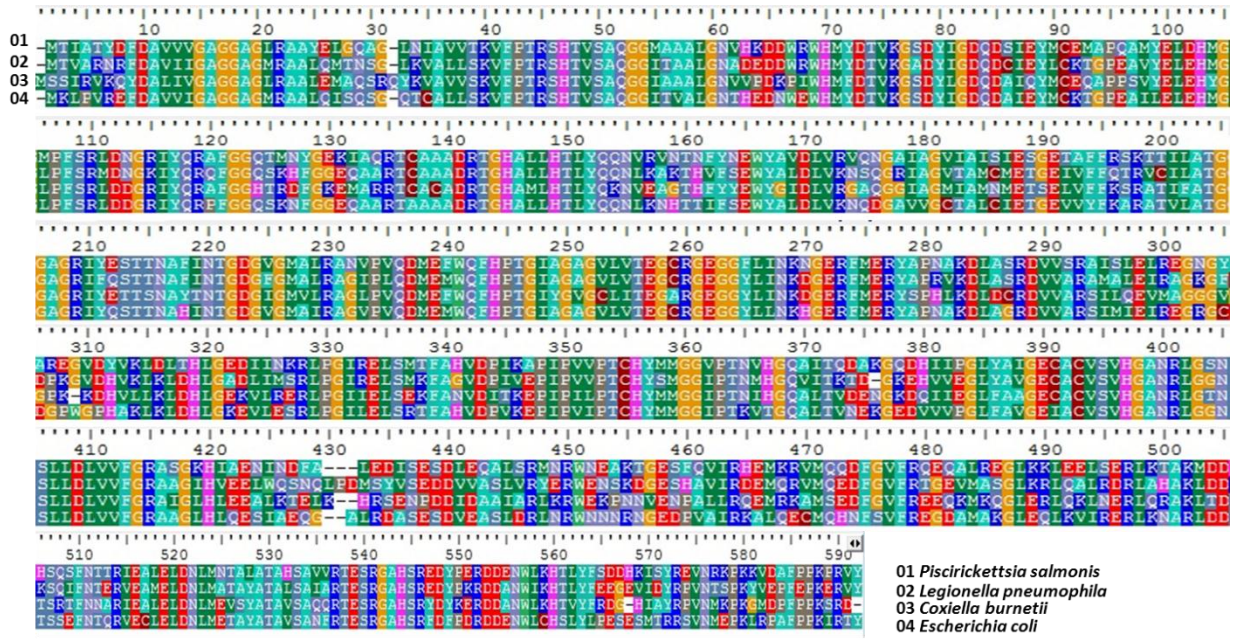

Figure 7: multiple alignment of SdhA aminoacid sequence from *P. salmonis* (01), *L. pneumophila* (02), *C. burnetii* (03) and *E. coli* (04).

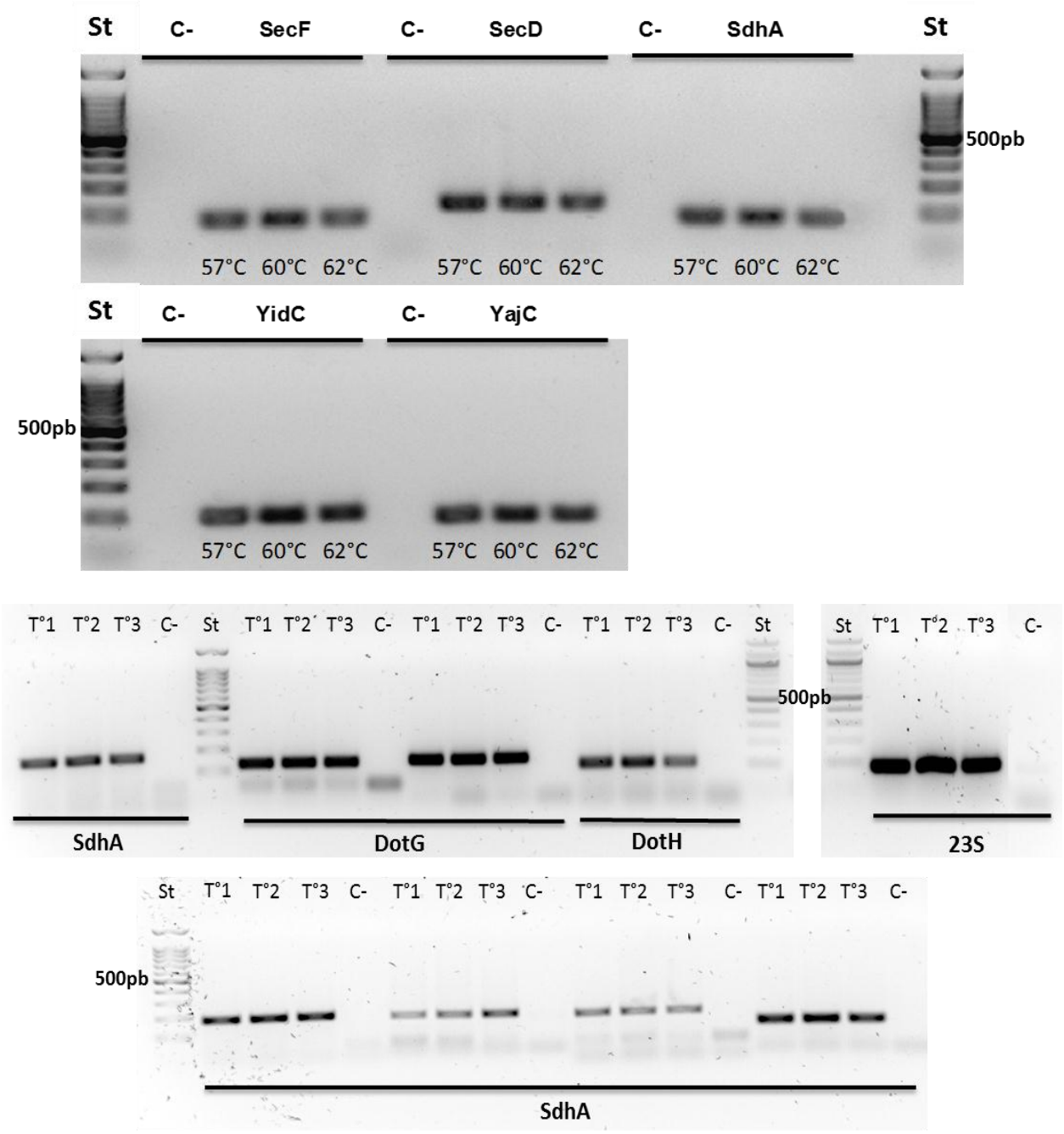

Figure 8: primer standardization using different annealing temperatures (T1: 57°C; T2: 60°C; T3: 62°C) to amplify the transcript from *secD, secF, yidC, yajC, dotG*, *dotH*, *sdhA* and *23S* genes in *Piscirickettsia salmonis*. The 23S primer was used to normalize and was specifically designed to detect *P. salmonis* strain AUSTRAL-005.

Table 1: primers used to detect the transcript of Sec-dependent pathway, T4BSS and *sdhA* genes.

Primer name	Sequence	Putative Gene
DotH	Fwd-TTACCAATGCTCCGGCAACT	*dotH/icmK*
	Rev- GCCGGTGAGATTAACGTTCC	
DotG	Fwd- AACAAGTGCAGCAACCGAGA	*dotG/icmE*
	Rev- GACCGTTGGTGGCCGATTA	
DotF	Fwd- CAATTATTACTGGGCGGGCTTG	*dotF/icmG*
	Rev- AACCCAGAGCTCGTGATAACTTT	
SdhA	Fwd- ATCGCGGTGGTAACGAAAGT	*sdhA*
	Rev- CATGTGCCAACGCCAATCAT	
SecD	Fwd- CGAGACTCCTGGTGGAAGGT	*secD*
	Rev- TCACATCAAAGCCACGACGA	
SecF	Fwd- CCAAAGCGCAGGCTTAAGTG	*secF*
	Rev- AACCGGTTGGCCTAATGTCA	
YajC	Fwd- TCCACAAAGTAAGCGTGCCA	*yajC*
	Rev- CACTTTACCCAACACACCGC	
YidC	Fwd- GGCAATTGCAGTATGGGCCT	*yidC*
	Rev- TCAGCAGATCGCGTGTTAGT	
23S	Fwd- TTGAAAACCGGTGTTGAGAT	*23S*
	Rev- CTCTAACTGCCAAGGCATCC	

3.3 RELATIVE QUANTIFICATION

Once the primers were standardized, they were used to evaluate the expression levels of the Sec-dependent pathway and the T4BSS genes during infectious process induced by *P. salmonis* in cell line SHK-1.

The putative genes from Sec-dependent pathway and T4BSS were amplified successfully using specific primers and the relative level of expression during an infectious process is shown in figure 9. It is very probable that both secretion systems can act together to translocate substrate into the cytosol of the host cell but it is needed to verify if the gene product is present during the infectious process induced in the same conditions used earlier.

Different patterns of gene expression were observed for each gene during *in vitro* infection. Genes belonging to the T4BSS increased at early times post infection, from 2hpi until 2dpi, showing a decreased expression after 4dpi. Genes belonging to Sec-dependent pathway showed increased levels from 8hpi showing a peak of increased expression at 24hpi continuing with high levels of transcripts until the end of the experiment at 6dpi. The expression of the auxiliary protein YidC showed a similar pattern like SecDF/YajC holotranslocon increasing from 8hpi until the end of the experiment.

The expression pattern for *sdhA* gene showed early increase from 8hpi with a peak at 4dpi to decrease after this since 6dpi but continuous higher than the calibrator point until the end of the experiment.

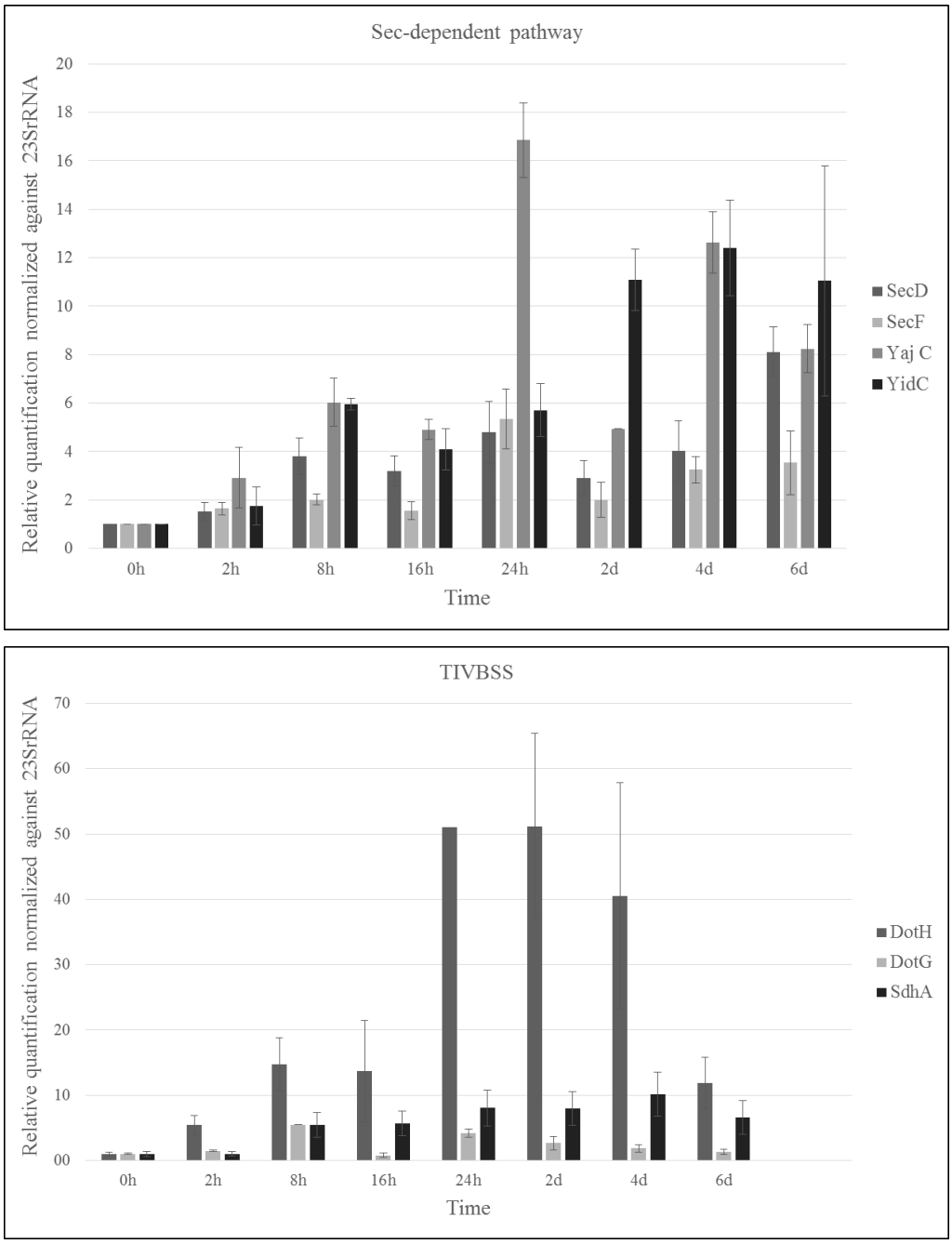

Figure 9: relative quantification of expression levels from *secD, secF, yajC, yidC* (upper panel) and *dotH*, *dotG* and *sdhA* (lower panel) genes during infectious process induced by *P. salmonis* in SHK-1 cell line normalized with *23S* gene.

3.4 ANTIBODY PRODUCTION

After the successful detection of specific transcripts from T4BSS, Sec-dependent pathway and effector protein genes, it was decided to generate polyclonal antibodies using the aminoacid sequence derived from nucleotide sequence from the same possible candidate genes *dotH/icmK, dotG/icmE* and *sdhA* from *P. salmonis* AUSTRAL-005 strain genome, to analyze them and look for the best antigen to design synthetic peptides.

In the beginnings of this research the sequence for these three proteins in this pathogen was not available. So the design was made by choosing the possible proteins of the Type 4B Secretion System described in *Legionella pneumophila* and *Coxiella burnetii*, comparing with *Piscirickettsia salmonis* AUSTRAL-005 strain genome. Currently, are available the annotated proteins described for this Secretion System in the complete genome of Lf-89 strain by Pulgar *et al.* (2015).

The aminoacid sequence analysis was made by online server using the Kolaskar and Tongaonkar (1990) method to predict antigenic determinants along with other parameters like hydrophilicity, surface accessibility and linear epitope prediction (fig. 10) to predict the best antigens to design synthetic peptides (table 2).

Once the synthetic peptides were synthetized they were analyzed against online protein database. The three synthetic peptides show between 75 to 100% of aminoacid sequence identity with the annotated proteins DotH, DotG and SdhA in *P. salmonis* (fig. 11) LF-89 strain.

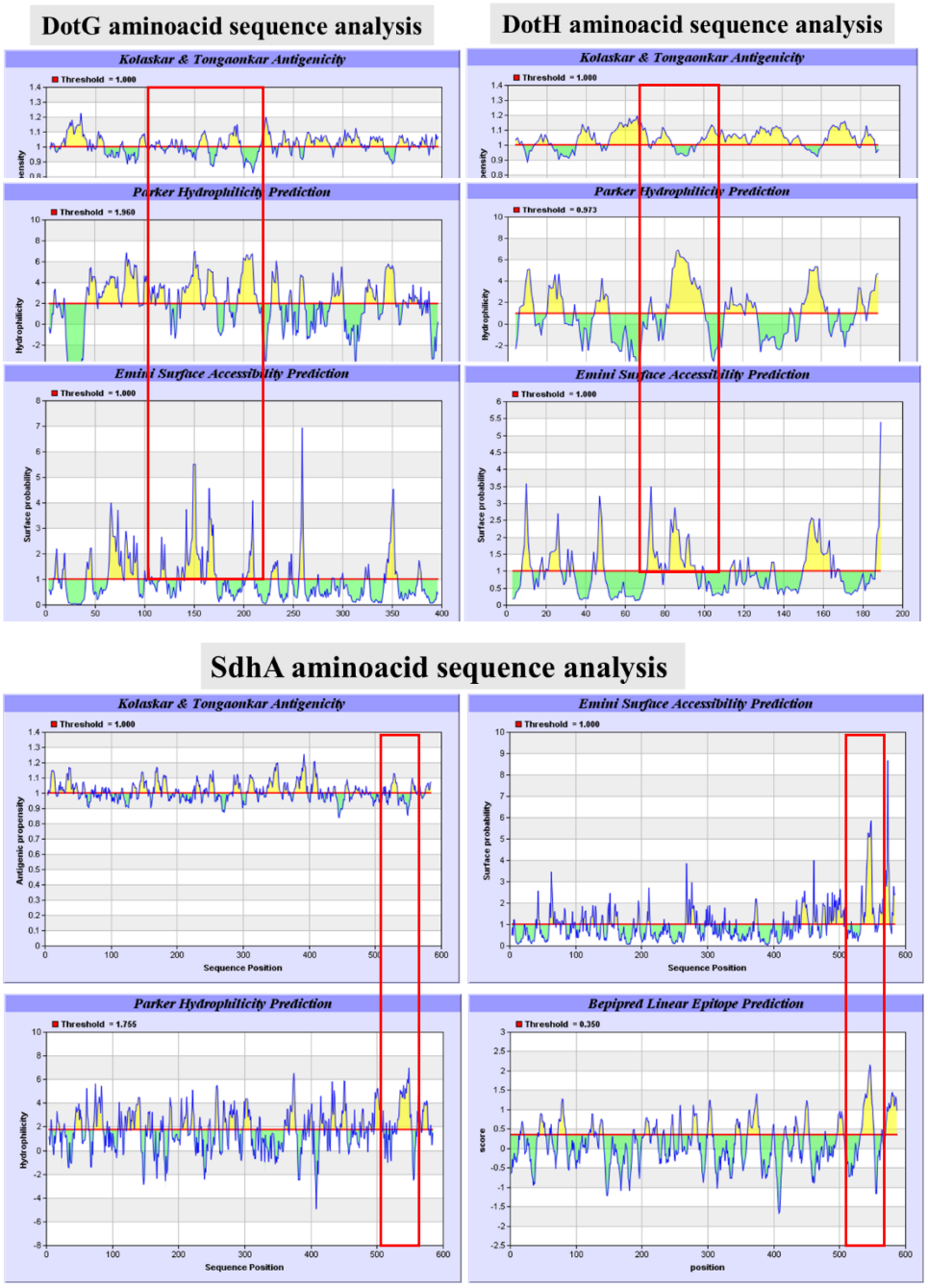

Figure 10: aminoacid sequence analysis of DotG, DotH and SdhA proteins to look for the best antigens of each protein for synthetic peptide design. The red boxes show approximately the position of the synthetic peptides that were made.

Table 2: synthetic peptides designed to generate polyclonal antibody in animal model.

PROTEIN NAME	SYNTHETIC PEPTIDE	ACC. NUMBER
DotH	LSGVLMVQGIKPYGQT	A0A0B8UZN4
DotG	NHQNLQEEKEQQEVDQYQLKVS	A0A0B8V3N1
SdhA	KHTLYFSDDHKIS	A0A0B8UP92

Synthetic peptides chosen

>TrEMBL|A0A095BT71|Release 2015_03/2015_03
MKKCRLAKLFTIIFFSVLGCSYGSLSDDSFNSVVNNNFPLTTDQIQKYKEIYNEQKKAKAAPSGNAPSESTSSIISASLHPGDIEPVIR
TFKGMITSIVMTDQLGKVWPIISYSLGDPASFNIQWNK**TSGVLMVQGLKDYGQA**NIGIMLKGLDIPVMLSLVLGQKKWDYLDY
IRVQSYQSAADALAGTTPHAPSNLIQLLNGIPPQGAKELKVTGGSAQVWSYQGKYLLLTMGTLISPQWQAKQSSTGPSASNAYQL
PAAPSLLISMNGVLHNITVADS

Synthetic Peptide Number 1: **LSGVLMVQGIKPYGQT**

>tr|A0A0B8UP92|A0A0B8UP92_PISSA Succinate dehydrogenase flavoprotein subunit OS=Piscirickettsia salmonis GN=KW89_00972 PE=3 SV=1
MTIATYDFDAVVVGAGGAGLRAAYELGQAGLNIAVVTKVFPTRSHTVSAQGGMAAALGNVHKDDWRWHMYDTVKGSDYIGD
QDSIEYMCEMAPQAMYELDHMGMPFSRLDNGRIYQRAFGGQTMNYGEKIAQRTCAAADRTGHALLHTLYQQNVRVNTNFYNE
WYAVDLVRVQNGAIAGVIAISIESGETAFFRSKTTILATGGAGRIYESTTNAFINTGDGVGMALRANVPVQDMEFWQFHPTGIAGA
GVLVTEGCRGEGGFLINKNGERFMERYAPNAKDLASRDVVSRAISLELREGNGYAPEGVDYVKLDLTHLGEDIINKRLPGIRELS
MTFAHVDPIKAPIPVVPTCHYMMGGVPTNVHGQAITQDAKGQDHIIPGLYAIGECACVSVHGANRLGSNSLLDLVVFGRASGKHI
AENINDFALEDISESDLEQALARMNRWNEAKTGESFQVIRHEMKRVMQQDFGVFRQEQALREGLKKLEELSERLKTAKMDDHS
QSFNTTRIEALELDNLMNTALATAHSAVVRTESRGAHSREDYPERDDENWL**KHTLYFSDDHKIS**YREVNRKPKKVDAFPPKPRV
Y

Synthetic Peptide Number 2: **KHTLYFSDDHKIS**

>tr|A0A0B8V979|A0A0B8V979_PISSA Type IV secretion protein IcmE OS=Piscirickettsia salmonis GN=KW89_00207 PE=4 SV=1
MKLKAITKTKEFLSEHPRLRVLIIFAIIMLVIIFIVNNLSQPKSAEEVGGSYVASPNGAMSQKTTKYTNTTYKKIHKANEDEEVNRA
ENQGKTLVASSTQDISPRAPGAPELSQADRRIQELTGSHIFKEMVQREAIQK**NHQNLQEEKEQQEVDQYQLKV**SEKENKMQNK
IQGILAKWENVSAQKVMVAKVSTVNNAGDNGQSGKNNSENIIEKAGAIVFAVLDTQLNSDQPGTPVMATIVQGKFKNAKLLGSF
KREDEKLVISFDRMSLPELDHSISIKAYAINATTAQNALSSDVDNHYLLRYGGLFAAAFLQGFGDYFSQNSSSLCGGATTCIITGTQS
TAEQNRTTKKALYSGLGQVGTTLAGKASAAFDRPPTVTLNQGVGMGILFMSDVKV

Synthetic Peptide Number 3: **NHQNLQEEKEQQEVDQYQLKV**

Figure 11: aminoacid sequence of DotH, SdhA and DotG from database showing the synthetic peptide (highlighted in red) synthetized to generate polyclonal antibodies.

The main purpose was to generate polyclonal antibodies against DotG, DotH and SdhA proteins from *P. salmonis* AUSTRAL-005 strain.

After three months of inoculation with synthetic peptides in two different animal models (hens and rats) the polyclonal antibodies from hen yolk and rat sera for all three proteins DotH, DotG and SdhA from *P. salmonis* were obtained.

Then their characterization was started through dot blot and western blot assays comparing them with the sera and yolk previous to the immunization with the synthetic peptides as a control antibodies (pre-immune sera). These antibodies were used against the AUSTRAL-005 and LF-89 strains growing in liquid medium to detect the described DotH, DotG and SdhA proteins in *L. pneumophila* and *C. burnetii*.

The results show the best dilution (1:250 primary antibody) for each polyclonal antibody detecting several protein bands in those samples from liquid medium (figure 12).

For the two strains used of *P. salmonis*, positive reaction for DotH and DotG was detected at the relative theoretical mass derived from the aminoacid sequence, 21kDa and 45kDa respectively; the only antibody that reacted in a different relative theoretical mass with approximately 50kDa was SdhA protein (65kDa theoretical).

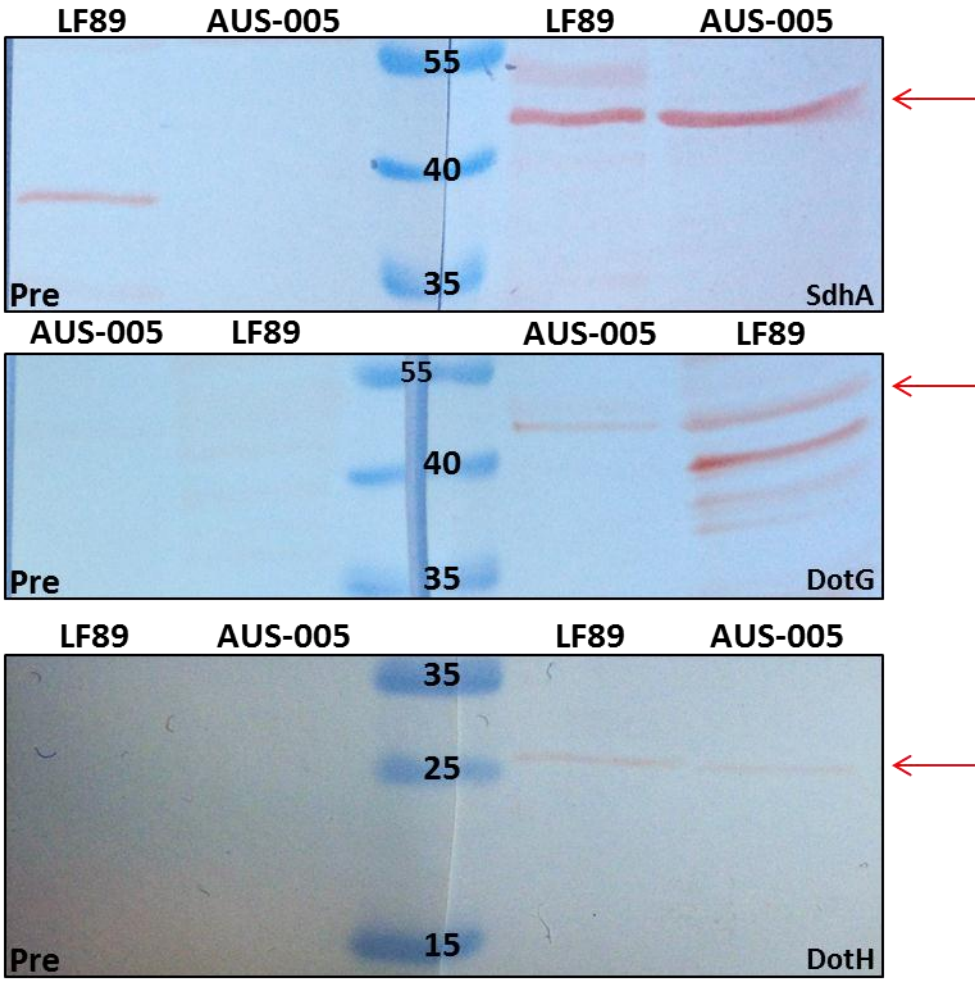

Figure 12: immunedetection of SdhA, DotG and DotH (red arrows in each panel) proteins with polyclonal antibodies. The bacterium was cultured in AUSTRAL-SRS broth and the samples were extracted at fifth day post inoculation in fresh medium. The dilution for each polyclonal primary antibody was 1:250 and the specific positive signal was detected in both strains at the same amount of total protein in each lane (40μg).

3.5 PROTEIN DETECTION BY POLYCLONAL ANTIBODIES

Using total protein extracts from *P. salmonis* AUSTRAL-005 strain, a proteome analysis was made to visualize the whole range of proteins that exists in the bacteria growing in liquid medium at exponential phase. From this result it was found that around 15% of these proteins are involved in other process than metabolism and those without determined function. And inside this type of other processes the transport processes are found which are involved the Type 4B Secretion System among others (fig. 13).

To prove this statement it was looked for the presence of the Dot/Icm proteins, Sec-dependent pathway and the effector protein SdhA in the bacterial proteome. It was really great to find almost all the proteins described to T4BSS from *L. pneumophila* and *C. burnetii* in the proteome of *P. salmonis*. Likewise the proteins described to Sec-dependent pathway and the effector protein SdhA (table 3).

The core proteins of the T4BSS are present in the proteome, namely DotH, DotG, DotF, DotC and DotD, as well as, the effector protein described in *L. pneumophila,* SdhA. Also other important proteins in the same secretion system are present like chaperones and ATPases. For the Sec-dependent pathway the core complex that forms the SecDF-YajC holotranslocon together with SecB and SecA was found (table 3).

Using the standardized antibodies already made, denaturant electrophoresis in polyacrylamide gels (SDS-PAGE) was performed in order to separate the total protein extract samples to analyze for the presence of DotH, DotG and SdhA during *in vitro* infection induced by *P. salmonis* in SHK-1 cell line (figure 14). Silver staining was used to verify the protein pattern in each lane. The total protein amount in each lane was 20μg.

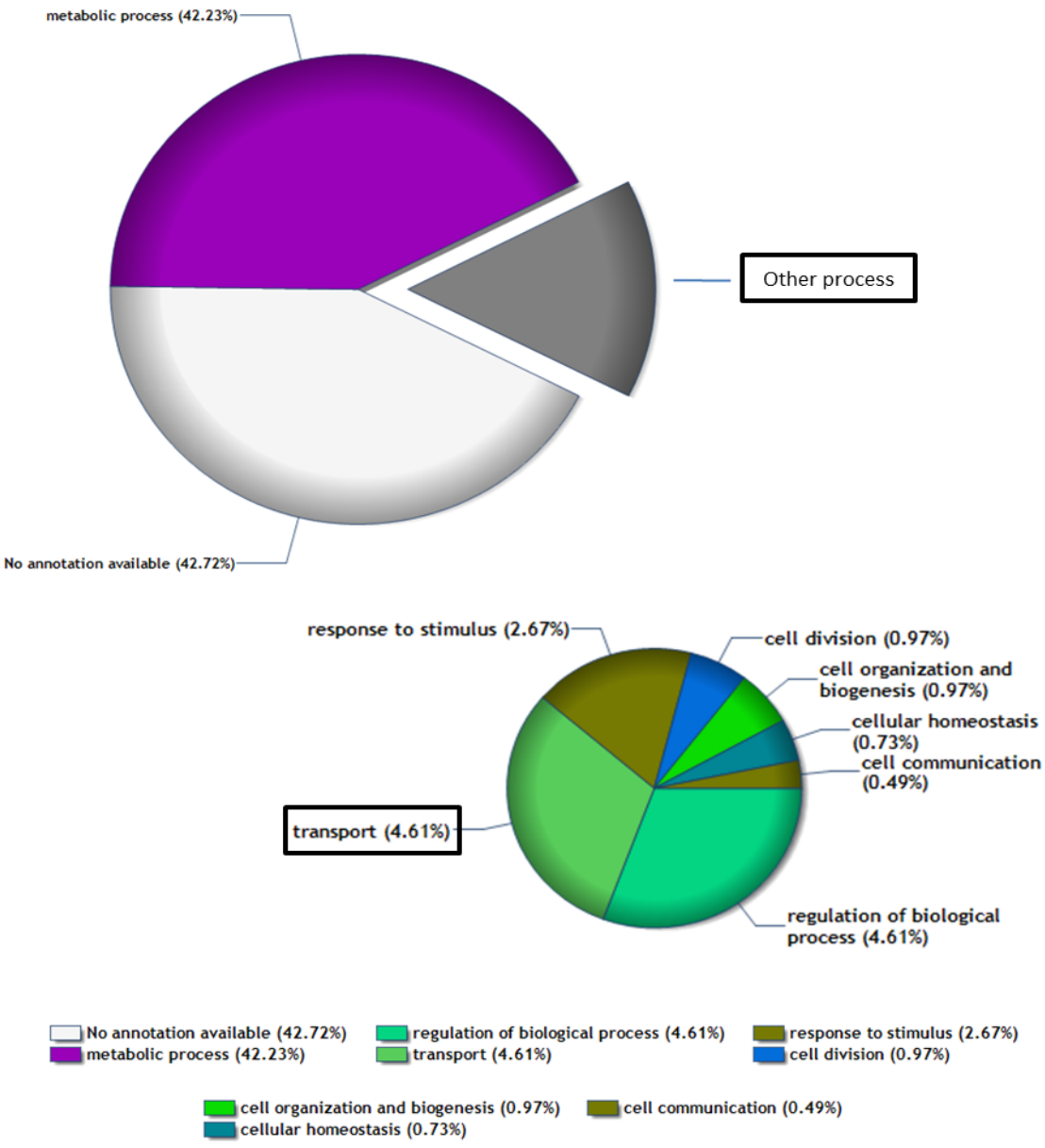

Figure 13: Proteome analysis of *Piscirickettsia salmonis* AUSTRAL-005 strain. Here is shown the proteome analysis of the AUSTRAL-005 strain where are highlighted the proteins involved in the transport mechanism which probably should be present the Type 4B protein Secretion System among others.

Table 3. Proteome analysis of *P. salmonis* showing the proteins belonging to the Type 4B Secretion System, Sec-dependent pathway and effector protein (Dot/Icm, Sec proteins and SdhA respectively). Score, times detected, coverage, percentage of protein found, #peptides, number of peptides from the detected protein. The proteins DotH, DotG and SdhA are highlighted.

Acc. Number	Description	Score	Coverage	# Peptides
A0A095BUI2	DotD	11,03	97,02	10
A0A095CP73	DotB	2,56	76,6	17
A0A095BKQ6	IcmW	2,33	66,43	7
A0A095BT71	**IcmK/DotH**	11,75	65.59	12
A0A095DSE8	IcmO	7,53	48,44	33
A0A095BIW8	DotA	14,79	40,52	24
A0A0B8V6U7	IcmG/DotF	5,73	64,12	26
A0A0B8V979	**IcmE/DotG**	4,23	42,07	25
A0A0B8UR39	IcmB	4,99	41,87	55
A0A0B8UG95	**SdhA**	30,56	50,43	44
A0A0B8UK28	SecA	20,00	44,18	64
A0A0B8UNG4	**SecD**	9,69	34,89	29
A0A0B8V1U5	**SecF**	3,87	27,07	17
A0A0B8UVN9	SecB	2,02	20,12	3
A0A0B8V9Y9	**YajC**	6,65	24,79	4

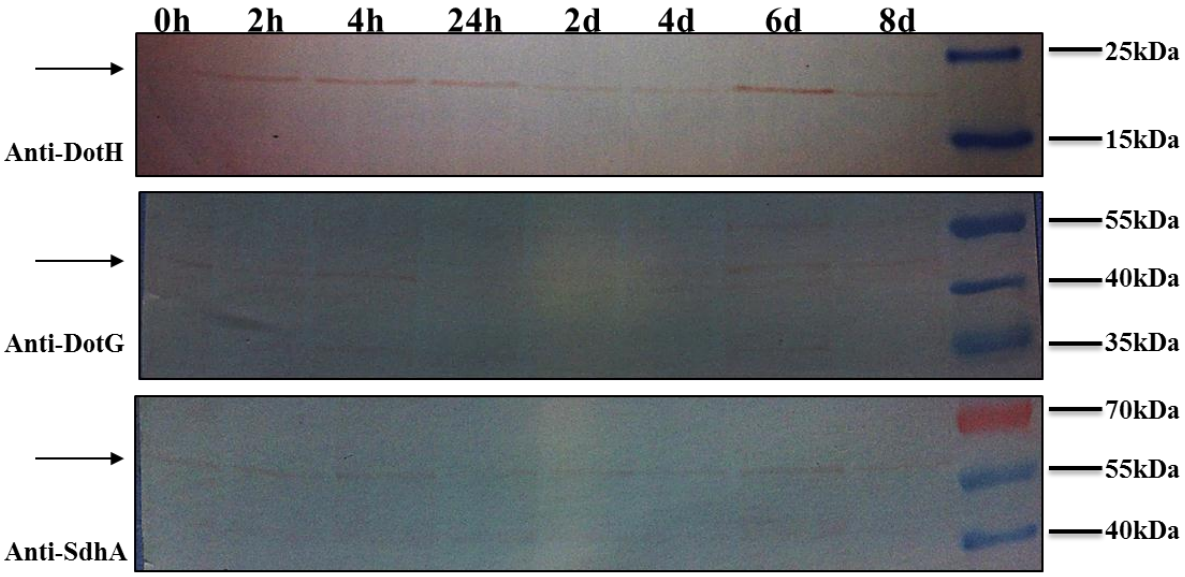

Figure 14: Western blot analyzes detecting DotH, DotG and SdhA from *P. salmonis* during an infectious process in SHK-1 cell line (20 μg total protein each lane).

3.6 ANTISENSE OLIGONUCLEOTIDE ASSAY

After the detection of transcripts and proteins DotH, DotG and SdhA, it was aimed to determine the possible function of SdhA protein during *in vitro* infection induced by *P. salmonis*. First at all, the aminoacid sequence of SdhA protein from *P. salmonis* (described in *L. pneumophila*) was analyzed by online programs that predict effector proteins using several parameters described for Yejun *et al.* (2014). The algorithm used was able to predict around 81% of *Legionella* effectors among them the Type 4 effector proteins. With this training data, the aminoacid sequence of SdhA from *P. salmonis* was analyzed comparing it with other proteins related to T4BSS. Among these proteins, PmrA is a gene regulator protein for T4BSS described in *Legionella* and *Coxiella* (Zusman *et al.*, 2007), and in this case it was used as a negative control for effector protein. The results show that SdhA from *P. salmonis* was identified as an effector protein as well as the positive control VipE (effector protein described in *Legionella*), compared to PmrA protein from *P. salmonis,* that was found negative as an effector protein as expected (table 4).

Before this analysis was done, it was found that this pathogen, like other Gram-negative bacteria, produces Outer Membrane Vesicle (OMV). Here it is demonstrated by Oliver *et al.* (2015), that this pathogen produces OMVs when is growing in liquid medium and during an *in vitro* infection (fig. 15). In figure 15 in panels B, C, E and F is visualized the OMV production. In B and E TEM images from the formation and release of this vesicles and in C and F SEM images that show the form and the approximated size of the OMVs produced by *P. salmonis*. In the same image 15, is visualized the OMVs during *in vitro* infection (black arrow head) near to the bacteria (Ps). The size of these OMVs is variable with a mean size of 45 nm.

Inside of these OMVs there are a lot of proteins, RNA and DNA, all of which "try" to modify the environment to its own benefits, like a first step of infection establishment. In this context, effector proteins like SdhA should be present inside this vesicle. To support that statement, the proteome of a sample of these OMVs was determined and the presence of a few peptides belonging to the SdhA protein from *P. salmonis* was found, proving that this protein should be an important factor during infection.

Table 4. SdhA from *P. salmonis* as an effector protein predicted by T4SEpre and T4EffPred. None of the chaperons (HtpB and Hsp60) were identified as a Type 4 effector protein (negative controls). T4SEpre utilize algorithm SVM (Support Vector Machines) based on training data to classify the new data. A large amount of effector proteins from T4SS used to training the program. This algorithm was able to identify 81% of *Legionella* effectors (Yejun *et al.*, 2014). The red box shows the positive result for SdhA from *P. salmonis* as an effector protein from T4SS. The PmrA gene regulator protein from *P. salmonis* was negative as an effector protein, as expected.

	T4SEpre		T4EffPred
Protein	SVM value	T4SS Effector	T4SS Effector
LpVipE	1.0001370865	YES	YES
LpHtpB	-1.3705154117	NOT	NOT
PsHsp60	-0.9963425828	NOT	NOT
PsSdhA	1.0003162116	YES	YES
PsPmrA	-0.9972815317	NOT	NOT

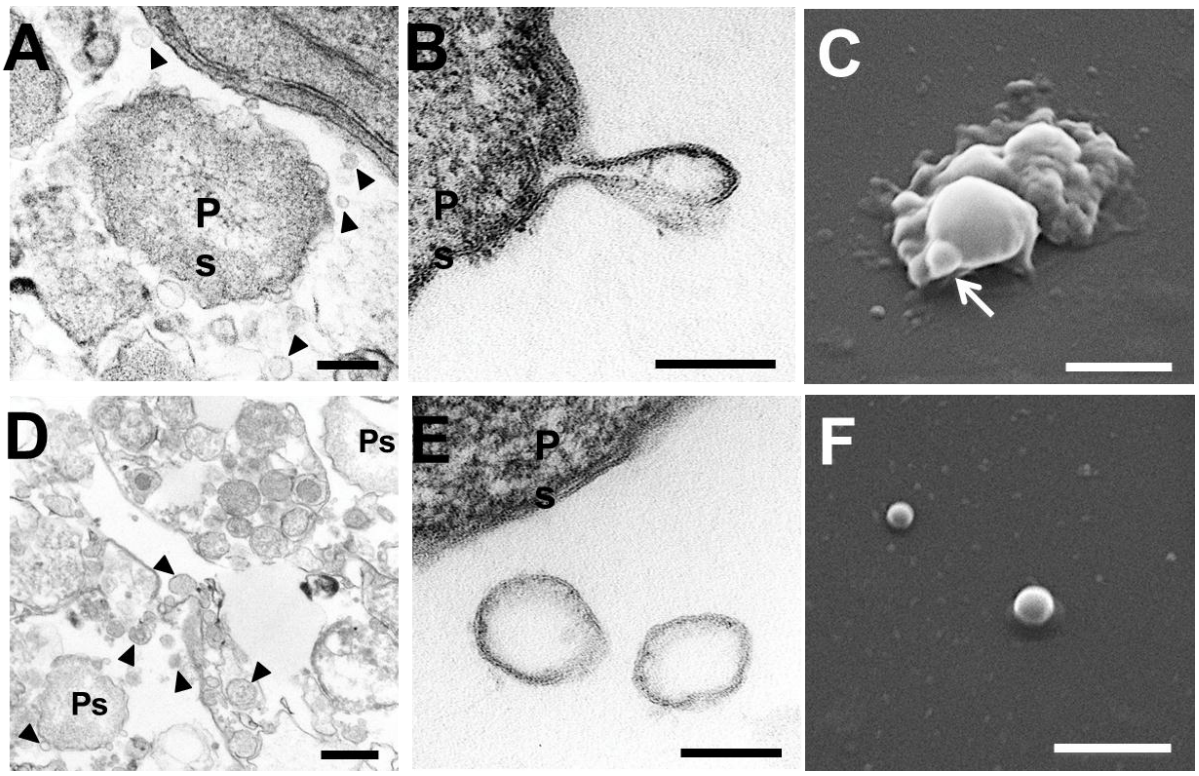

Figure 15: Production of Outer Membrane Vesicles (OMVs) from *P. salmonis*. Transmission Electron Microscope images (A and D) of OMVs from *P. salmonis* (Ps) infecting SHK-1 cells (head arrows), and also is shown the formation (B) and released (E) of OMVs from bacterium. Scanning Electron Microscope (SEM) images shown the shape and approximately the size of the OMVs produced by *P. salmonis* (Oliver *et al.*, 2015).

The OMV proteome was determined to look for the presence of the SdhA inside this vesicle. The results show that twelve peptides aligned with the aminoacid sequence of SdhA from *P. salmonis* (Table 5).

Doing further analysis with *P. salmonis* AUS-005 genome, a gene regulator protein PmrA was found that regulates the gene expression of T4BSS and some T4SS effector proteins described for *Legionella* and *Coxiella* by Zusman *et al.* (2007). Using the nucleotide sequence of PmrA, a phylogenetic analysis was done to compare it with other bacteria as well as with *sdhA* gene. The nucleotide sequence of *pmrA* gene from *P. salmonis* was located near to *Legionella pmrA* gene sequence (fig. 16).

Then, a multiple alignment between PmrA proteins from *P. salmonis* LF-89, *C. burnetii, E. coli, L. feeleii* and *P. salmonis* AUS-005 (fig. 17) was calculated. In all the sequences the motif described by Zusman *et al.* (2007), for PmrA, LEVHIHNLR was found (red line below the sequence in fig. 17).

Table 5. SdhA peptides sequence from *P. salmonis* OMVs proteome identified from trypsin-digested gel by LC-MS/MS.

Peptide sequence	XCorr[a]	m/z [Da][b]
LGSNSLLDLVVFGR	3,58	1490,01482
AFGGQTMNYGEK	3,38	1303,84353
HTLYFSDDHK	3,26	1262,49448
LDLTHLGEDIINK	3,06	1481,49991
TGESFQVIR	3.06	1037,83694
GEGGFLINK	2,80	934,50104
VMQQDFGVFR	2,78	1227,67498
MDDHSQSFNTTR	2,74	1440,68026
KLEELSER	2,11	1003,50275
EGNGYAPEGVDYVK	1,50	1499,84839
QEQALREGLK	2,07	1171,50692
VMQQDFGVFRQEQALR	1,42	1952,51159

[a] SEQUEST correlation scores (XCorr) are indicated.
[b] The values represented in the m/z (Da) gives the precursor mass of the identified peptides.

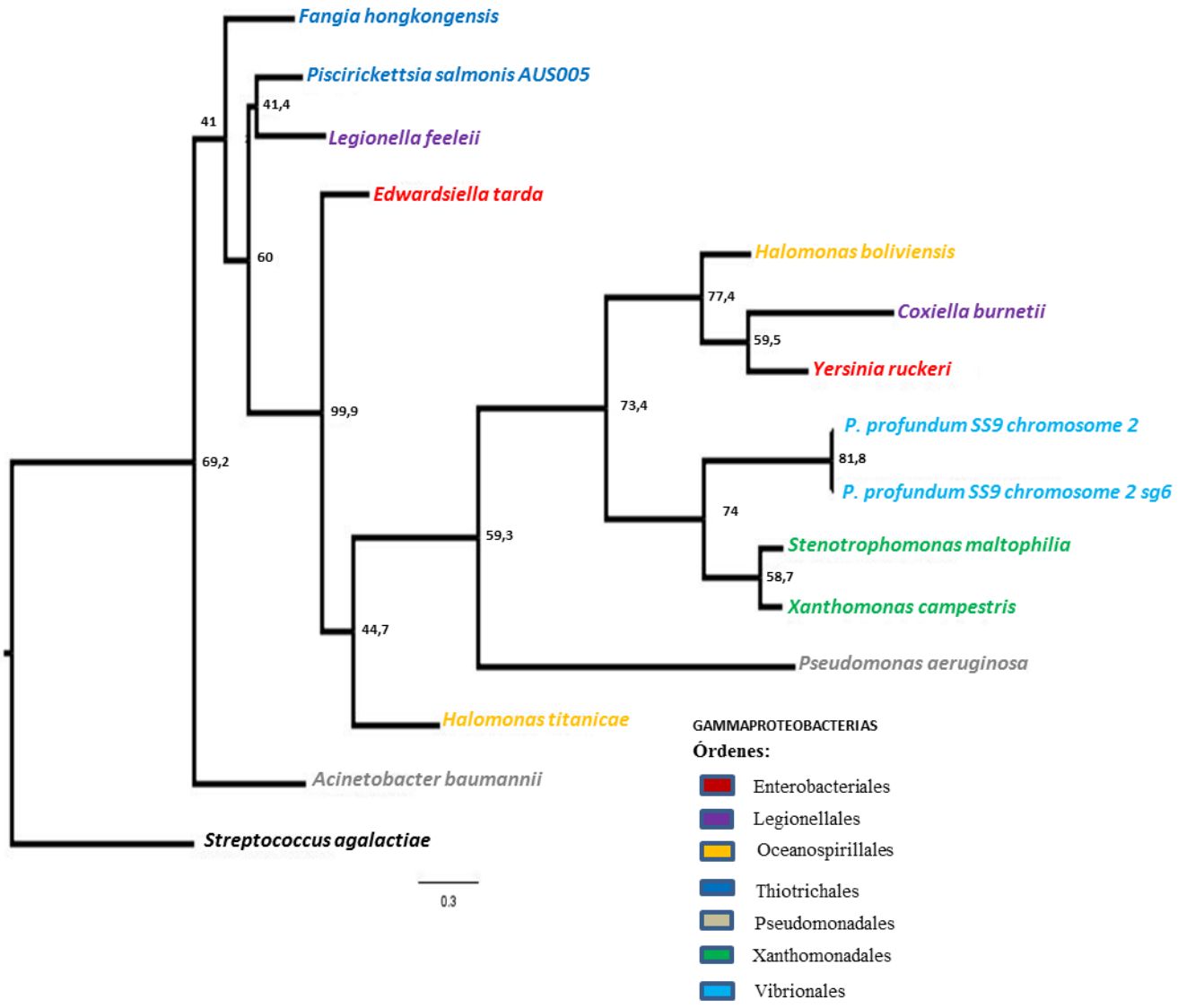

Fig. 16: Phylogenetic analysis of *pmrA* gene from *P. salmonis*. Nucleotide sequence analysis of *pmrA* genes described in Gammaproteobacteria belonging to different orders.

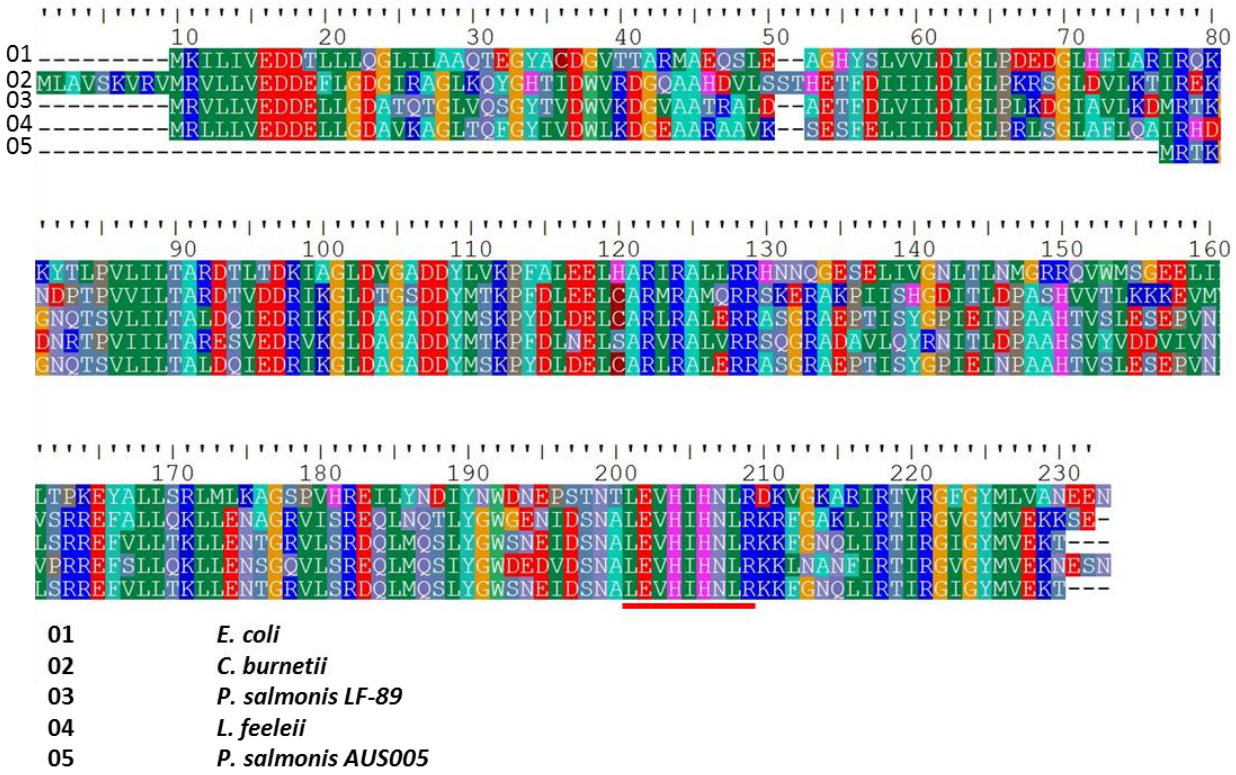

Fig. 17: Multiple alignment using aminoacid sequence of PmrA proteins described for several bacteria compared with *Piscirickettsia salmonis* Austral-005 strain. The described conserved motif for PmrA protein (LEVHIHNLR) is underlined (Zusman, et. al., 2007).

Now, after all this previous analysis, and using the nucleotide sequence of the T4BSS genes and the effector protein SdhA, it was used to designed Antisense Oligonucleotides (ASO) to inhibit the translation of the specific messenger RNA coding for *sdhA, dotH, dotG* and *pmrA* genes to decrease the protein amount in *Piscirickettsia salmonis*. An example of the ASO design is shown in figure 18.

Table 6 summaries the ASOs that were used for this experiment. The ASO was designed to block the ribosome binding site in each mRNA for the specific gene; electroporation was used to deliver it inside the bacteria. With this type of ASO design, western blot analysis were carried out to evaluate the protein amount shift using polyclonal antibodies to detect the presence of DotH, DotG and SdhA (fig 19). The absence of each protein when the specific ASO was used to inhibit the specific mRNA is not completely clear. The presence of each protein in each control, and also in the same samples using silver staining (data not shown), however indicates that presumably the ASO worked at least in some cases to inhibit the translation of the specific mRNA.

>SdhA-*P. salmonis*

5'UCUGG**GGUGUAUAAUUCAUGACAAU**UGCAACAUAUGAUUUCGACGCCGUUGUCGUGGGCGCCGGUGGGGCU
GGUUUGCGUGCGGCCUAUGAGUUAGGGCAAGCGGGCUUAAAUAUCGCGGUGGUAACGAAAGUCUUUCCAACAC
GUUCACAUACAGUGUCAGCGCAAGGGGGCAUGGCCGCGGCUCUUGGUAAUGUUCAUAAAGAUGAUUGGCGUUG
GCACAUGUACGAUACCGUCAAGGGGUCUGAUUAUAUCGGUGAUCAGGACUCUAUUGAGUAUAUGUGUGAGAUG
GCACCGCAAGCCAUGUACGAGCUUGACCAUAUGGGUAUGCCGUUCUCGCGCUUGGAUAAUGGUCGCAUCUAUCA
GCGUGCAUUUGGUGGUCAGACCAUGAAUUACGGUGAGAAAAUCGCUCAGCGUACUUGUGCGGCAGCAGAUCGU
ACCGGCCAUGCCUUAUUGCAUACGCUGUAUCAGCAAAAUGUGCGGGUGAAUACUAAUUUUUAUAACGAAUGGU
AUGCUGUUGAUUUGGUGAGAGUGCAAAACGGUGCAAUCGCCGGUGUGAUUGCCAUUUCAAUAGAAAGUGGUG
AAACCGCCUUUUUCCGUUCAAAAACGACGAUUCUGGCUACCGGUGGGGCGGGGCGUAUCUAUGAAUCAACCACC
AAUGCCUUUAUUAAUACCGGCGAUGGUGUGGGGAUGGCUUUGCGGGCGAAUGUCCCUGUGCAAGAUAUGGAG
UUCUGGCAAUUUCACCCAACCGGCAUUGCUGGUGCGGGUGUCUUAGUGACAGAAGGUUGUCGUGGUGAAGGC
GGCUUUUUAAUUAAU 3'

ASO-SdhA: **GGTGTATAATTCATGACAAT**

Fig. 18: antisense oligonucleotides (ASO) design example. Using the nucleotide sequence to *dotH, dotG* and effector protein (*sdhA* gene), the antisense oligonucleotides were aim to the ATG site and the 5' end sequence to block the ribosomal binding site and therefore block the translation of the mRNA.

Table 6: Antisense Oligonucleotides (ASO) to inhibit the translation of the specific messenger RNA.

GENE	ANTISENSE OLIGONUCLEOTIDES	ACC. NUMBER
dotH	ATGATTACATCGATTGTAAT	CP011849.1
dotG	GTATTTGCTGTACTTGATAC	CP011849.1
sdhA	GGTGTATAATTCATGACAAT	CP011849.1
Control	GCTAAGCATTACACCATTTG	AC239108.3

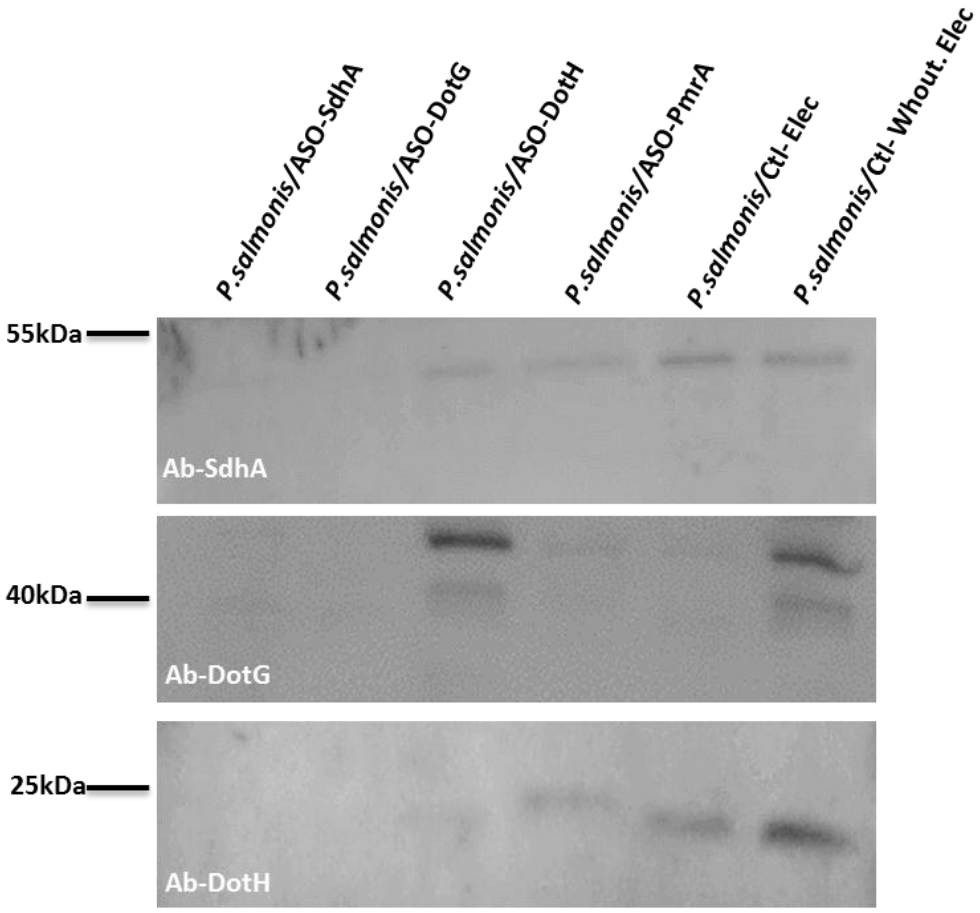

Figure 19: Western blot analyses to prove the translation inhibition of the SdhA, DotG and DotH proteins induced by the ASO treatment. Samples: *P. salmonis*/ASO-SdhA; *P. salmonis*/ASO-DotG; *P. salmonis*/ASO-DotH; *P. salmonis*/*ASO-PmrA; P. salmonis*/ASO-Control Antisense; *P. salmonis*/Control without electroporation. Total amount of protein extract in each lane was 80 μg. Silver staining of the same samples was used as a loading control (data not shown).

4. DISCUSSION

Using the available genome of *P. salmonis* AUSTRAL-005 strain, sequenced in our laboratory, it was analyzed for the putative genes belonging to bacterial protein secretion systems. At the moment of writing, genes for almost all the secretion systems were found, but just the Type 4B Secretion System (T4BSS) was nearly complete compared with the T4BSS described for *L. pneumophila*. The proteins belonging to the core of the T4BSS were complete so those proteins were further analyzed. With the currently latest assembled nucleotide sequence, the T4BSS genes were identified in three contigs. Only one contig (136) evidenced all the Dot/Icm genes described for *L. pneumophila* and *C. burnetii*, supporting strongly the idea that this secretion system could be acting during the infection process (fig 6). In agreement with the latest article published by Pulgar *et al.* 2015, they also described three copies of the same T4BSS genes in the genome of *P. salmonis* LF-89. The redundancy of the gene copy amount of this secretion system is a disadvantage energetically speaking; but otherwise it is advantageous when the bacteria are infecting cells, so the RNA polymerase can easily find the genes and transcribe them (Klappenbach *et al.*, 2000). The presence of multicopy genes in bacterial genomes contributes to a better adaptation to environmental stress (Kondrashov *et al.*, 2002).

Also found were genes belonging to General Secretion Pathway (GSP or type 2 secretion system), specifically the Sec-dependent pathway (fig. 6) described in *E. coli* (Xie and Dalbey, 2008). This Sec-dependent pathway also plays a prominent role in protein export and secretion in bacteria. It is also worth to note that all the components forming the secretion apparatus from type 1 to 6 secretion system are membrane inserted or exported by the Sec system (Dalbey and Kuhn, 2012).

It is of importance to have found and to analyze the Sec-dependent pathway and the T4BSS in this fish pathogen to help us to understand the behavior of this bacterium and how we can design new strategies to avoid the disease in salmon farming.

Continuing with the search of possible key factors in bacterial genome, a possible effector protein in *P. salmonis* AUSTRAL-005 strain was found. Using conserved motifs between effector proteins described in *L. pneumophila* and *C. burnetii,* and among other bacteria in the NCBI database, the *sdhA* gene (fig. 6) was found, described as a key protein in *L. pneumophila*, involved in the integrity maintenance of vacuolar membrane inside the host cell (Creasey and Isberg, 2011). The aminoacid sequence of SdhA derived from the nucleotide sequence found in *P. salmonis* was used for a multiple alignment with the respective SdhA aminoacid sequences described for *L. pneumophila, C. burnetii* and *E. coli*. The similarity between these sequences is very high around 70% at least and makes one suppose that perhaps this protein has the same function in *P. salmonis* (fig. 7). One of the mechanisms proposed to SdhA in *Legionella*, beside to maintain the integrity of the membrane vacuole, is to inhibit the apoptosis to allow the bacteria to grow inside the host. In fact, Wall and McCormick (2014), sum up that bacterial infection has the ability to manipulate the activation or inhibition of programmed cell death. There are two possible ways to inhibit the apoptotic pathway. One of them is the inhibition of Caspase-3 using some yet unidentified T4SS substrate or mediating the activation of pathways that prevent the Caspase-3 activity. In both processes, the bacteria prolong the time to replicate inside the host cell to ensure its survival. Studies using the human pathogen *Legionella pneumophila* with a mutated SdhA protein showed that this bacterium cannot establish the infection inside the cell (Creasey and Isberg, 2011). So, if this protein is present in *P. salmonis* during *in vitro* infection, SdhA could act in the same manner like it does in *Legionella*.

Inhibition of the SdhA translation perhaps could stop or delay the progression of *in vitro* infection. Maybe, if the translation could be blocked successfully by using ASO and then infect the cells with this treated bacteria, the viability of this microorganism would be less than pathogenic bacteria without ASO treatment, because when the *Legionella* SdhA protein is truncated or depleted this bacterium cannot grow inside the cell (Isberg *et al.*, 2009).

At this moment, the detection of the proteins during the progression of *in vitro* infection give us a clue to begin to understand how the bacteria establish the infection and the key factors that this pathogen used to subvert all the enzyme mechanism to its own benefit.

According to the qRT-PCR results, the expression levels of *sdhA* gene during *in vitro* infection suggest that this protein could be acting during this process.

Further results from our laboratory looked for the expression levels of *sdhA* together with the expression levels of *caspase-3* genes, during an *in vitro* infection; the pathogenic bacteria showed increased *sdhA* gene expression levels compared with decreased *caspase-3* gene expression levels. On the other hand, when the bacteria were inactivated the *sdhA* gene expression levels are decreased and *caspase-3* are increased (unpublished data).

The gene for effector protein SdhA was also found overexpressed (fig. 9) during *in vitro* infection starting very early (2h post infection) with a peak at around 8h post infection; this expression levels was constant during the whole experiment. This could be related to the function that T4BSS gene clusters are doing during the infection because at early times bacteria need to inject effector proteins to subvert the host cell mechanism to its need and establish the infection in order to survive and replicate inside the vacuole. Although the gene expression levels for the T4BSS are not so striking, they are correlated with the gene expression levels of SdhA, and this effector protein is described as a T4SS substrate in *L. pneumophila* (Laguna *et al.*, 2006).

The gene expression levels for the Sec-dependent pathway perhaps are correlated also with the protein export of another substrate from T4SS that remains unidentified (fig. 9). One other explanation could be the increased amount of replicating bacteria inside the vacuole and their need to assemble the inner, outer and periplasmic space proteins. The Sec-dependent pathways are described to help in this function and also other types of secretion systems (Dalbey and Kuhn, 2012). The expression levels of the Sec-dependent pathway genes were different; perhaps because of the amount of proteins needed to assemble the holo-translocon SecDF/YajC (Schulze *et al.*, 2013). Also, for the nature of auxiliary protein YidC, the increased gene expression levels were observed during *in vitro* infection maybe are correlated to the function that this protein does during the replication of bacteria, translocating proteins from cytoplasm to inner membrane and/or periplasmic space by its own (Serek *et al.*, 2004).

Combining all the results until now, it can only be said that T4BSS and Sec-dependent pathway are functional, but until the presence of these proteins from those genes can be proven, this is only a speculation.

Pursuing this goal synthetic peptides were designed to generate polyclonal antibodies against DotH, DotG and SdhA proteins from *P. salmonis* AUSTRAL-005 strain.

When the antibodies were generated the pre-sera were taken as pre-immune sera as a control for each antibody and then each pre-immune/immune sera were tested individually. The best combination of pre-immune and immune sera was chosen to further experiments in western blot analysis.

The results showed that the detection of the proteins was successful. First of all, the proteins were immunodetected in liquid medium growing bacteria (fig. 12), and positive reactions for the three antibodies in *P. salmonis* LF-89 and AUSTRAL-005 strains were seen. The predicted relative mass for DotH protein (21 kDa) was detected between 20 – 25 kDa using this polyclonal antibody. The same positive reaction was seen with this polyclonal antibody against DotG protein (43 kDa), detecting a few bands between 40 – 45 kDa. And, for the polyclonal antibody against SdhA protein, showed positive reaction between 45 – 55 kDa, lower than the expected 65 kDa from the aminoacid sequence.

Once the proteins were detected in liquid medium growing bacteria, this result was compared with the proteome obtained from LF-89 strain. All the proteins belonging to T4BSS, Sec-dependent pathway and SdhA were found in the proteome (table 3). Moreover, the proteins for which polyclonal antibodies were made also were found in the proteome of LF-89 strain, corroborating the positive reaction in western blot analysis.

Another analysis using the aminoacid sequence of SdhA revealed that this protein could be substrate for an enzyme and the protein size detected in western blot comes from the proteolytic cutting. From the proposed action mechanism to SdhA from *Legionella* it is possible that this protein could be an inhibitor for apoptosis via Caspase (mentioned above), and its action blocks the functional site competing with the intracellular substrate to this enzyme, and inhibits the cell death (Laguna *et al.*, 2006; Creasey and Isberg, 2011; Wall and McCormick, 2014).

The immunodetection of DotH, DotG and SdhA during *in vitro* infection was also successful. The positive reaction can be closely related to gene expression levels because at early times post infection an increase in the level of the three proteins was seen as early as 2h post infection with a peak of positive signal between 8h and 16h, decreasing since 2d post infection but being still present in the whole infectious process (fig. 14). Although it will be better to have a loading control antibody specifically to this pathogen, the silver staining for all the samples showed an identical protein pattern during the whole experiment. With this result, it can be said that this T4BSS exists in *P. salmonis* and probably is involved to secrete effector proteins inside the host cell including SdhA protein during *in vitro* infection.

While the genes mentioned above were searched, also a gene regulator protein for the T4BSS named PmrA was found. This protein was described in *Legionella* to control the expression of almost all genes from T4BSS and some T4SS substrate (Zusman *et al.*, 2007). They mentioned that this gene regulator protein possess a conserved motif near to the carboxyl terminal (fig. 17). Looking at the result of this multiple alignment it can be seen that both *P. salmonis* AUSTRAL-005 and LF-89 strains have the same conserved motif described by Zusman and co-workers. Using the nucleotide sequence for this protein found in *P. salmonis* AUSTRAL-005 strain, a phylogenetic analysis comparing this *pmrA* gene with other bacteria was carried out (fig. 16). The nucleotide sequence was located near to *Legionella* and gives us a clue to its possible function for PmrA from *P. salmonis* like it does in this human pathogen.

Then, in an attempt to manipulate the bacteria, the Antisense Oligonucleotide (ASO) technique was used combined with electroporation to deliver the ASO inside the bacteria (fig. 18 and table 6).

Briefly, this technique is based on the hybridization between the messenger RNA with a small modified DNA sequence (like a primer) that targets this mRNA to inhibit the translation of a specific gene. For that, four specific ASOs against *dotH, dotG, sdhA* genes, and also against to this gene regulator factor described to *L. pneumophila* and *C. burnetii*, PmrA were designed (van Schaik E, *et al.*, 2013). For this experiment LF-89 and AUSTRAL-005 strains from *P. salmonis* growing in liquid medium were used.

The ASOs were designed to inhibit the translation of the specific genes covering the Shine-Dalgarno sequence at the ATG from each gene, and the way to prove if the experiment worked was by western blot analysis. After bacterial electroporation to get the ASO inside, bacteria were incubated for at least 5 days in liquid medium and the total protein was extracted to perform western blot analysis. The results showed (fig. 19) that all the samples from LF-89 strain were inhibited successfully; in the case of AUSTRAL-005 strain the experiment was successful for DotH and DotG ASO (fig. 19). Perhaps for the SdhA ASO in the AUSTRAL-005 strain, the design of the ASO was not very specific and the ASO couldn't find the mRNA target during the time of the experiment. For the DotH and DotG ASO it should be noted that those genes have three copies each in the genome, so the design of ASO must be correlated with the gene that shows a high level of transcription during *in vitro* infection. In this case, the ASO were directed against different contigs to each DotH and DotG, so probably the inhibition by ASO was poor or not completely efficient as was planned.

Now, it is worthy to note that in the case of PmrA protein, the translation of *dotH* and *dotG* genes were inhibited in some cases, perhaps because the ASO amount was not optimal. It could be the case that by improving the conditions for this experiments one could inhibit completely the translation of all the genes involved in the secretion system combining electroporation and ASO techniques.

5. CONCLUSIONS

- Genes from Sec-dependent pathway, T4BSS and SdhA effector protein are increased during *in vitro* infection induced by *P. salmonis*.
- Proteins from T4BSS DotH and DotG, plus the effector protein SdhA from *P. salmonis* are present during *in vitro* infection induced by *P. salmonis*.
- The ASO technique combined with electroporation represent a possibility to try to modify or manipulate the bacteria to study the possible function of certain genes/proteins from *P. salmonis*.
- All the results obtained in this thesis work proved that the proteins, DotH, DotG and SdhA, are important during *in vitro* infection induced by *Piscirickettsia salmonis*.
- With these results, a schematic model for the possible distribution of the T4BSS and Sec-dependent pathway in *Piscirickettsia salmonis* was proposed (figure 20).

6. PERSPECTIVES

Currently little is known about how the pathogen *Piscirickettsia salmonis* can establish the infection in fish and replicate inside the cells. The biggest challenge is to describe possible candidate proteins to develop new strategies to avoid the outcome of this disease and diminish the mortality induced by *Piscirickettsia salmonis*. Until now, the good management in salmon farming has been controlling the spread of the disease. The wrong and unusual use of antibiotics can produce the emergence of resistant bacterial causing this disease that can resist the treatment with useful antibiotics, thereby inducing big economic losses to the industry. This research contributes to describe the possible ways of how this pathogen can insert effector proteins inside the host cell and how it can replicate and survive during the whole progression of the disease. With this basic knowledge, we now can try to develop new kind of foods with different nutrients and/or small amount of antigens so the fishes can react against them to develop a better immunity response.

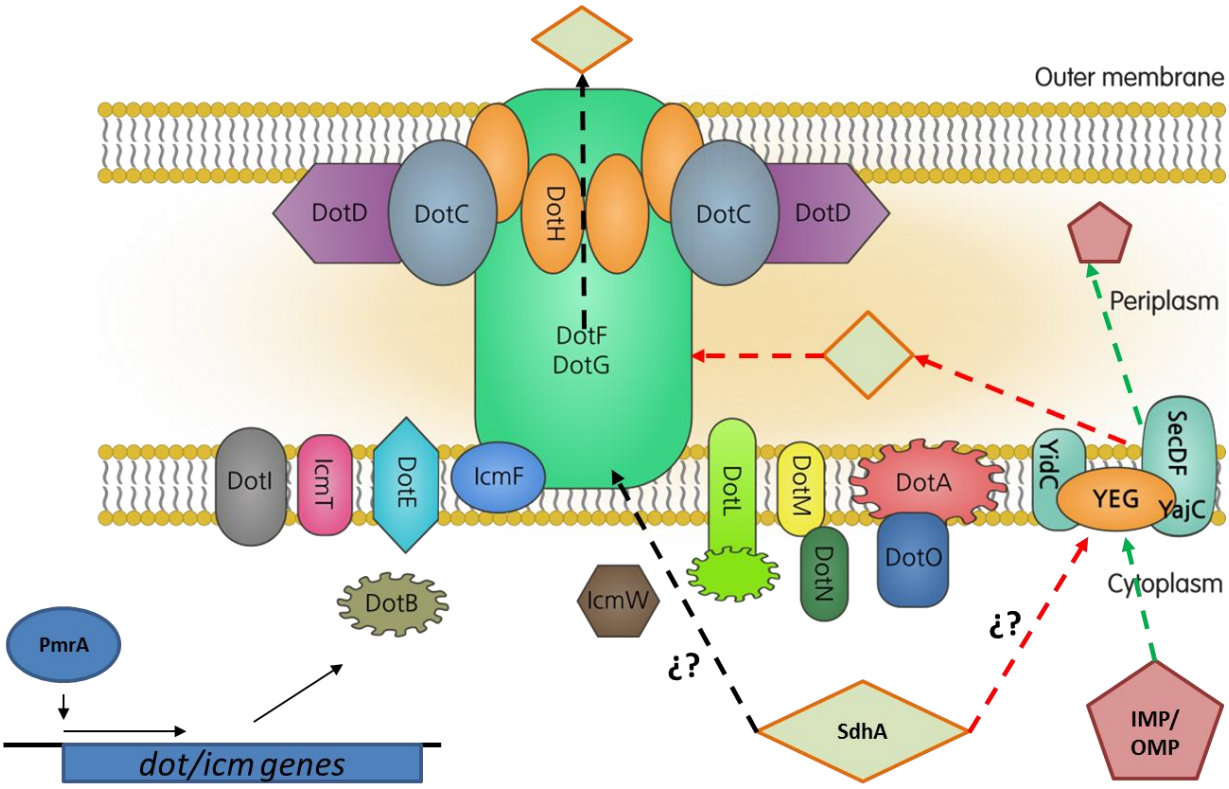

Figure 20: schematic model of the distribution of GSP and T4BSS proteins in *P. salmonis* with the possible substrate using proposed models by: Isberg, *et al.*, 2009; Schulze, *et al.*, 2013; van Schaik, *et al.*, 2013.

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